SCHOOL OF BIOLOGY, CHEMISTRY AND FORENSIC SCIENCE

BACTERIAL POLY-GAMMA-GLUTAMIC ACID (γ-PGA) – A PROMISING BIOSORBENT OF HEAVY METALS

A thesis submitted for the degree of Doctor of Philosophy

By

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DECLARATION

I declare that no material contained in the thesis has been used in any other submission for an academic award.

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ABSTRACT

Poly-γ-glutamic acid (γ-PGA) is a biopolymer made up of repeating units of L-glutamic acid, D-glutamic acid or both. γ-PGA is water soluble, non-toxic and biodegradable, and can be used safely in a variety of applications that are increasing rapidly. This study investigated the production of HMW γ-PGA by five *Bacillus* species (*B. licheniformis* 1525, *B. licheniformis* NCTC 6816, *B. licheniformis* ATCC 9945a, *B. licheniformis* ATCC 9945a and *B. subtilis* (natto) ATCC 15245) in GS, C and E media for the removal of heavy metals in wastewaters. The highest γ-PGA yields of 11.69 g/l and 11.59 g/l were produced by *Bacillus subtilis* (natto) ATCC 15245 in GS medium and medium C respectively. Upon characterization, γ-PGAs with different properties (crystallinity, acid/salt form and molecular weights ranging from 2.56×10^5 Da to 1.65×10^6 Da) were produced. The water soluble, non-toxic, HMW (M_w 1.65×10^6 Da) γ-PGA produced by *B. subtilis* (natto) ATCC 15245 in medium C was investigated as a sorbent for the removal of heavy metal ions including Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+} and Ag^{+}. The results showed that the removal of metals by γ-PGA was more dependent on the concentration of γ-PGA than the solution pH. The highest metal ions removal of 93.50%, 88.13%, 90.21%, 90.56% and 86.34% by HMW γ-PGA were obtained for Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+} and Ag^{+} respectively. The presence of interfering metal ions could hinder the adsorption of individual metal ions by γ-PGA. The affinities of heavy metal ions for γ-PGA followed the order: $Cu^{2+} > Zn^{2+} > Ni^{2+} > Cd^{2+}$. The effect of molecular weight of γ-PGA on metal removal was also investigated, and it was found that metal ion adsorption capacity of γ-PGA strongly depended on its molecular weight. The maximum amount (93.50%) of Cu^{2+} sorbed by HMW γ-PGA was higher compared to that (59.48%) sorbed by LMW γ-PGA. Isotherm models showed that the Redlich-Peterson best described the metal adsorption capacity of γ-PGA. It was also found that a multisite adsorption mechanism occurred via the complexation of metal ions with the free α-carboxyl and possibly the amide functional groups in γ-PGA.

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Dedicated to the Almighty God

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ABBREVIATIONS

Activated carbon	AC
Adenosine diphosphate	ADP
Adenosine monophosphate	AMP
Adenosine triphosphate	ATP
Alpha ketoglutaric acid	α-KG
Biological oxygen demand	BOD
Blast furnace slag	BFS
Chemical Oxygen Demand	COD
Coal fly ash	CFA
Colony forming units	CFU
Dilution factor	DF
Dimethyl sulphoxide	DMSO
Dispersity index	*Đ*
Dissolved-air flotation	DAF
Electrocoagulation	EC
Electrodialysis	ED
Electro-flotation	EF
European Union	EU
Extracellular polysaccharides	EPSs
Fourier Transform Infra-Red Spectroscopy	FT-IR
Gel permeation chromatography	GPC
Generally regarded as safe	GRAS
Glutamic acid	GA
Glutamyl-transpeptidases	GGT
High molecular weight	HMW
Inductively Coupled Plasma-Atomic Emission Spectroscopy	ICP-AES
Kelvin	K
Lethal toxin	LT
Low molecular weight	LMW

Molecular weight	M_w
Molecular weight	M_n
Nanofiltration	NF
National Centre for Biotechnology Information	NCBI
National Collection of Industrial and Marine Bacteria	NCIMB
Non-ribosomal peptide synthetases	NRPSs
Nuclear magnetic resonance spectroscopy	NMR
Phosphate ion	Pi
Polyelectrolyte complex	PEC
Polyglutamic acid	PGA
Polytetrefluorethylene	PTFE
Poly-α-glutamic acid	α-PGA
Poly-γ-glutamic acid	γ-PGA
Poly-γ-glutamic acid synthetase complex	pgsBCA
Pyrophosphate	PPi
Reverse osmosis	RO
Solid state fermentations	SSF
Sugarcane bagasse pith activated carbon	SBP-AC
Suspended Solids	SS
Thin Layer Chromatography	TLC
Tricarboxylic acid	TCA
Tryptone soy agar	TSA
Tryptone soy broth	TSB
Ultrafiltration	UF
United States Environmental Protection Agency	USEPA
World Health Organisation	WHO
X-Ray Diffraction	XRD
γ-glutamyl-hydrolase	PgsS

BACKGROUND OF STUDY

Water is the most essential constituent of life and water scarcity is becoming an increasing problem due to rapid industrial growth, environmental pollution and depleted water resources among others (Lee and Park, 2013 and Qu *et al*., 2013). Human activities play a great role in increasing water scarcity by contaminating natural water sources both in developing and industrialized countries (Qu *et al*., 2013). The main sources of water pollution include storm water and urban runoff, wastewaters from domestic (municipal), agricultural and industrial activities (Chiu *et al*., 2015). In as much as the development of manufacturing technology after industrial revolution had improved the standards of living significantly, water pollution is again becoming an issue that is threatening human health and the environment (Lee and Park, 2013).

The presence of hazardous and toxic pollutants such as heavy metals and organic compounds in industrial wastewater is one of the most serious environmental problems the world is faced with today (Kyzas and Kostoglou, 2014). Heavy metals are mostly challenging because they are toxic, non-biodegradable and can be accumulated in the environment and living tissues, causing various diseases and disorders (Tang *et al*., 2014 and Witek-Krowiak *et al*., 2011). Heavy metals such as chromium, cobalt, nickel, copper, zinc, silver, cadmium, mercury enter the water streams through both natural and human-induced sources. The natural sources include erosion, weathering of minerals and volcanic activity while electroplating, smelting, textile production, mining, battery manufacturing, tanneries, petroleum refining, and industrial discharge are the major human-induced sources of heavy metal contamination (Ali *et al*., 2013 and Ahmaruzzaman, 2011).

Hence, the potential hazard of heavy metal pollution has led to an increased interest in developing systems to clean up the waters and even sewages. There are several technologies that use physio-chemical processes such as precipitation, dialysis or ion-exchange resins, electro-coagulation and membrane filtration to remove contaminants from polluted

environments (Hua *et al*., 2012, Nguyen *et al*., 2013 and Tang *et al*., 2014). Most of these technologies have several drawbacks such as high operation cost, low selectivity and/or production of large amount of sludge for disposal (Tang *et al*., 2014 and Witek-Krowiak *et al*., 2011).

However, in recent years, adsorption has become a common method for the removal of heavy metals from aqueous solutions. It seems to be one of the most efficient methods for heavy metal removal from wastewater due to its flexibility in design and operation, generation of high-quality treated effluent, availability in wide range and the possibility of adsorbent regeneration by suitable desorption processes for multiple use (Ali, 2012, Hua *et al*., 2012 and Witek-Krowiak *et al*., 2011). Commonly used adsorbents include agricultural wastes, industrial by-products, natural materials and biological material "biosorbents" (Witek-Krowiak *et al*., 2011). Biosorption involves the ability of active sites on the surface of biomaterials to bind and adsorb heavy metals from aqueous solutions and can be used to treat wastewater with low heavy metal concentrations as a simple, economical and effective unconventional method (Witek-Krowiak *et al*., 2011). Agricultural waste products, natural polymers, living and non-living micro-organisms and extracellular polysaccharides (EPSs) secreted by microorganisms can be used as potential biosorbents for the removal of heavy metals (Bulgariu and Bulgariu, 2012, Fomina and Gadd, 2014 and Witek-Krowiak *et al*., 2011).

More recently, the use of biopolymers as biosorbents in the removal of heavy metals from aqueous solutions have received wide attention because their non-toxicity, biodegradability, metal sorption capability and wide material sources, such as bacteria. The presence of several charged surface functional groups which can effectively bind metal ions in biopolymers make them potential biosorbents (Bodnár *et al*., 2013 and Wang *et al*., 2014). One of such biopolymers is poly-gamma-glutamic acid (γ-PGA). Poly-γ-glutamic acid is an edible, water soluble, non-toxic and biodegradable polyamide rich in amide and carboxyl groups (Wang *et*

al., 2014). γ-PGA exhibits good flocculating ability and binds metal ions with high affinity. As a result, γ-PGA and its derivatives exhibit applications in the area of water treatment (Bodnár *et al*., 2013). This study investigates the removal of heavy metals by γ-PGA. Chapter 1 will introduce heavy metals in industrial wastewater and methods for their removal while chapter 2 will present γ-PGA, a potential biosorbent in the removal of heavy metals from aqueous solutions.

1.0 INTRODUCTION TO REMOVAL OF HEAVY METALS

1.1 Introduction

Water pollution can be defined as any change in the physical, chemical and biological properties of water that has a detrimental effect on living things (WHO 1997). Water pollution is a major environmental problem in many developing countries (Gambhir *et al*., 2012). Industrial activities such as production of different chemicals, production of fuel and energy, mining and smelting of metalliferous as well as food and dairies, plastics, pharmaceuticals, electroplating and metallurgicals have led to a vast quantity of liquid wastes being distributed to the environment (Wang and Chen, 2009 and Kieu *et al*., 2011). Among these wastes, heavy metals have aroused more concern considering their persistence, bio-magnification and toxicity (Tang *et al*., 2014).

According to Duffus (2002), the term heavy metals is an inappropriate term that lacks articulate scientific explanation, nevertheless, the term is usually used to refer to a group of metals and semi-metals that have been linked to biological contamination and toxicity. Koul *et al*. (2012) defined heavy metals as "any metallic elements that have relatively high density and are toxic even at low concentration" whereas, Singh *et al*. (2011) has described 'heavy metal' as any toxic metal regardless of their atomic mass or density. They include zinc (Zn), copper (Cu), nickel (Ni), mercury (Hg), cadmium (Cd), lead (Pb), arsenic (As) and chromium (Cr) are often detected in industrial wastewaters and are of great concern due to their toxicological effect on human and other forms of life as reported in **Table 1.1**.

Some heavy metals like Zinc and Copper are required by living organisms in trace amounts, but are detrimental when in excess. Excessive amount of zinc results in serious health problems including skin irritations, nausea and anaemia, whereas in trace amount, it controls a large number of biochemical processes and is vital for the physiological function of living tissues (Fu and Wang, 2011). Copper is required in animal metabolism. However, when copper is consumed in excess, it leads to threatening health conditions such as vomiting, stomach cramps and convulsions or eventual death as a result of its toxic effect.

The toxicity of heavy metals can persist for a long period of time in nature and arises in concentration of about 1.0–10 mg/L and even in lower concentration of 0.001–0.1 mg/L in highly toxic metal ions, like Hg^{2+} and Cd^{2+} as shown in **Table 1.1**. Excessive amounts of some heavy metals substitute and/or displace ions from biomolecules and obstruct the functional groups of significant molecules such as essential nutrient transport systems and enzymes thereby altering enzymes' structure as well as inactivating them, disordering the composition of the cell membrane and causing damage to the nerves, liver, bones and DNA structure (Wang and Chen, 2006, Kieu *et al*., 2011 and Sunitha *et al*., 2013). Nickel is said to be carcinogenic to human and results in gastrointestinal distress, weight loss, skin dermatitis and pulmonary fibrosis as well as damages the liver and heart (Fu and Wang, 2011 and Gautam *et al*., 2014). The toxicity of some heavy metals depends on their environment, as such species (e.g. mercury) switches from a low to high toxicity under certain environmental conditions such as solid surfaces adsorption and complexation. The United States Environmental Protection Agency (USEPA) has identified Cadmium as a possible carcinogen since its persistent and extreme exposure leads to renal dysfunction and death respectively (Fu and Wang, 2011). Chromium causes mere skin irritation and in high levels could lead to more life threatening health problems such as lung carcinoma while lead damages the central nervous system as well as the liver, kidney, brain functions and the reproductive system (Fu and Wang, 2011 and Gautam *et al*., 2014). Heavy metals unlike organic wastes cannot be degraded by any technique, not even biologically but can be transformed from toxic species to less toxic species (Kieu *et al*., 2011).

Due to the mobility and toxicity of heavy metals as well as their usefulness, treatment to remove and/or recover them from wastewater before release into the environment is therefore necessary. **Table 1.1** below presents the sources, permissible limits and health effects of various toxic metals.

Table 1.1: Sources, health consequences and permissible limits of heavy metals

Pollutants	***Sources***	***Toxicities***	Permissible limits for industrial effluent discharge (mg/L)	Permissible limits for potable water (mg/L)				References
			WHO	**EU Standard**	**EU community**	**US EPA**	**WHO**	
Zinc	Metal processing, electro-plating, plumbing, coal-fired power generation, refineries and brass manufacture	Anaemia, depression, lethargy, neurologic signs such as seizures and ataxia, abdominal pain and increased thirst	5.0 - 15.0	-	5	5.0	3.0	Sud *et al*., 2008; Ahmaruzzaman, 2011; Ali *et al*., 2013; Gautam *et al*., 2014
Copper	Mining operations, metal processing and coal-fired power generation	Vomiting, stomach cramps, convulsions, liver damage, Wilson disease, insomnia or even death.	0.05–1.5	2.0	3	1.3	2.0	Sud *et al*., 2008; Ahmaruzzaman, 2011; Ali *et al*., 2013; Gautam *et al*., 2014
Nickel	Electroplating operations, electronics, and metal cleaning industries	Dermatitis, nausea, chronic asthma, coughing, human carcinogen, severe damage of lungs and kidney.	-	0.02	0.1	0.1	0.02	Sud *et al*., 2008; Ahmaruzzaman, 2011; Ali *et al*., 2013; Gautam *et al*., 2014
Mercury	Pesticides, batteries and paper industry	Rheumatoid arthritis, damage to nervous system, protoplasm poisoning and neurotoxic.	-	0.001	0.001	0.002	0.001	Sud *et al*., 2008; Ahmaruzzaman, 2011; Ali *et al*., 2013; Gautam *et al*., 2014

Cadmium	Welding, paints, electroplating, pesticide fertilizer, nuclear fission plant	Kidney damage, renal disorder, Itai-Itai, bronchitis, human carcinogen.	0.1	0.005	0.2	0.005	0.003	Sud *et al*., 2008; Ahmaruzzaman, 2011; Ali *et al.*, 2013; Gautam *et al*., 2014
Lead	Paint, pesticide, smoking, automobile emission, mining, burning of coal, battery manufacture	Liver, kidney, gastrointestinal damage, mental retardation in children and probable human carcinogen.	0.1	0.01	0.5	0.1	0.01	Sud *et al*., 2008; Ahmaruzzaman, 2011; Ali *et al.*, 2013; Gautam *et al*., 2014
Chromium	Electroplating industry , tanneries, steel industries	Carcinogenic to human, nausea, vomiting, headache and diarrhoea, hair loss	-	0.05	0.5	0.1	0.05	Sud *et al*., 2008; Ahmaruzzaman, 2011; Ali *et al.*, 2013; Gautam *et al*., 2014
Arsenic	Pesticides, wood preservatives, metal smelters	Bronchitis, dermatitis, carcinogenic, causes melanosis, keratosis, liver tumours and hyperpigmentation in humans	-	0.01	0.01	0.01	0.01	Sud *et al*., 2008; Ahmaruzzaman, 2011; Ali *et al.*, 2013; Gautam *et al*., 2014

1.2 Conventional techniques for heavy metal removal from wastewater

Wastewaters constitutes approximately 90% of the huge amount of hazardous wastes generated as a result of the numerous activities of natural, industrial and domestic processes (Mantzavinos *et al*., 1999). Wastewater as defined by Raschid-Sally and Jayakody (2009) and Naidoo and Olaniran (2013) most often comprises of one or more of storm runoff, surface run-off liquids and waterborne solids from industrial activities, domestic sewage, commercial establishments that cannot be released in untreated form into water bodies such as lakes or streams due to public health, environmental and economic concern. Wastewater treatment is the process whereby these pollutants are removed from wastewater before discharge into receiving water bodies. Potable water (absence of toxic chemicals and pathogens) is of great importance to the world's health, and is also a critical requirement in a variety of key industries. Consequently a number of researches have been carried out in recent years to develop effective water treatment processes. Furthermore, with increased strictness on environmental regulations, special attention is now given to wastewater treatment in most industries.

As a result, a number of methods for removing heavy metals from aqueous solutions have been investigated. Conventional physicochemical methods include: chemical precipitation or neutralization, chemical oxidation or reduction, electrochemical treatment, filtration, ion exchange, evaporation, membrane separation techniques (electrodialysis, ultra-filtration and reverse osmosis) and activated carbon adsorption (Fu and Wang, 2011, Hua *et al*., 2012, Ali *et al*., 2012, Nguyen *et al*., 2013 and Tang *et al*., 2014). However, most of these methods are usually limited due to various disadvantages (**Table 1.2**). For instance, membrane techniques, ion exchange and adsorption by activated carbon cannot be used on a large scale as they are highly expensive when used to treat low concentrations of heavy metals in a large volume of contaminated water (Inbarag *et al*., 2009 and Wang and Chen, 2009). Electrochemical

treatment and chemical precipitation are also not effective as they generate large amounts of sludge that is difficult to treat and incapable of treating solutions containing metal ion concentrations within the range of 1 to 100 mg/l (Wang and Chen, 2009).

Table 1.2: Conventional treatment methods for metal removal

Treatment techniques	*Advantages*	*Disadvantages*
Chemical precipitation and filtration	Simple operation Cheap Good removal of most metals	Required for higher concentrations. Difficult separation Resulting sludges Additional cost for sludge disposal
Membrane filtration technologies	Good removal of heavy metals Requires small space	Concentrated sludge production Costly operation due to membrane fouling
Oxidation	Rapid process for toxic pollutants removal	High energy costs and formation of by-products
Reverse osmosis	Pure effluent for recycle	High pressure Membrane scaling High cost
Ion exchange	Metal recovery possible Metal selective	Expensive resins Absorbent requires regeneration or disposal Less number of heavy metal ions removed removal
Adsorption using activated carbon	Flexibility and simplicity of design, Insensitivity to toxic pollutants High metal binding capacities	No regeneration Requires activated carbon Performance is dependent on the adsorbent
Coagulation/flocculation	Economically practicable	High sludge production Formation of large particles
Electrochemical treatment	Metal recovery Rapid process and effective for certain metal ions No chemical consumption	For high concentrations High energy costs and formation of by-products
Evaporation	Pure effluent (for recycle)	Energy intensive Expensive Resulting sludges

Sources: Ahmaruzzaman, 2011 and Nguyen *et al*., 2013

1.2.1 Chemical Precipitation

Chemical precipitation generally involves a chemical reaction whereby precipitant agents react with dissolved heavy metal ions to form insoluble metal precipitates (Fu and Wang, 2011). The resulting precipitates can then be removed from the water either by sedimentation or filtration after which the treated water is decanted and discharged accordingly. The efficiency of chemical precipitation process depends on factors like concentration of ionic metals present in the solution, presence of other constituents that may inhibit the process of precipitation and the precipitant used (Oncel *et al*., 2013). Chemical precipitation method is usually in two forms – hydroxide precipitation and sulphide precipitation.

Hydroxide precipitation

Hydroxide precipitation is the most extensively used type of chemical precipitation due to its availability, simplicity, low-cost equipment requirement and ease of pH control (Oncel *et al*., 2013 and Barakat, 2011) as pH control is a significant criterion in hydroxide precipitation. Upon adjustment of pH to 9.0 – 11.0, the solubilities of several metal hydroxides are greatly reduced thereby, heavily improving metal removal (Kurniawan *et al*., 2006a and Fu and Wang, 2011). Lime (calcium hydroxide) is the most widely used hydroxide because it is inexpensive and available in most countries (Kurniawan *et al*., 2006a, Barakat, 2011 and Fu and Wang, 2011). Addition of caoagulating agents such as iron salts, alum and organic polymers can significantly improve the efficiency of heavy metal removal in the hydroxide precipitation method (Fu and Wang, 2011). Despite the vast usage and several advantages of hydroxide precipitation, it produces a large quantity of sludge that requires further treatment (Kurniawan *et al*., 2006a) and the presence of complexes and chelating agents in the wastewater will prevent metal hydroxide precipitation (Huisman *et al*., 2006 and Fu and Wang, 2011). Also, since some metal hydroxides are amphoteric, hydroxide precipitation might be ineffective

where there are different metals because the optimal pH for one metal may increase the solubility of the other (Fu and Wang, 2011).

Sulphide precipitation

Sulphide precipitation is also a type of chemical precipitation with high heavy metal removal efficiency. Sulphide precipitation, even though it has several advantages over lime precipitation, it also has potential risk associated with it (Huisman *et al*., 2006 and Fu and Wang, 2011). Its advantages include very low solubilities of metal sulphide precipitates as compared to hydroxide precipitates and non-amphoteric sulphide precipitates resulting in high rate metal removal over a wide range of pH unlike the hydroxide precipitation. Sulphide precipitation is almost insensitive to the presence of complexes and chelating agents and generates more dense and stable sludges that are capable of dewatering and therefore needs no further treatment (Huisman *et al*., 2006).

However, because heavy metal ions are usually in acid form, sulphide precipitants under acidic conditions can give rise to the production and emission of toxic H_2S fumes. It is thus vital that this process be carried out under basic or neutral condition (Fu and Wang, 2011).

1.2.2 Coagulation and flocculation

Coagulation involves the aggregation of insoluble and/or dissolved organic particles to form large aggregates (Renault *et al*., 2009). The coagulation process neutralises the charges that keep colloidal particles apart thereby destabilising them. Once the force responsible for separating them is neutralised, the suspended particles have the ability to stick together and can be subsequently removed by sedimentation, filtration and flotation processes (Fu and Wang, 2011, Simate *et al*., 2012 and Saritha *et al*., 2015). Many commonly used coagulants such as aluminium sulphate, ferric chloride and ferric sulphate have been employed in wastewater treatment processes and have effectively removed impurities and particulates by neutralising

the particles' charge. pH adjustment is required for this technique. When inorganic coagulants like ferric or aluminium salts are used in wastewater treatment, it is necessary to determine the optimum pH range in which metal hydroxide precipitates as the addition of metals to the wastewater decreases its pH. When the pH is near neutral, turbidity, suspended solids (SS) and chemical oxygen demand (COD) are greatly reduced (Renault *et al*., 2009). Coagulation is however limited by the inability to control the nature of the hydrolysis species formed when the coagulant is added to the solution (Bratby, 2006) and generation of a large quantity of sludge (Simate *et al*., 2012). The residual aluminium salts in the treated water also causes Alzheimer's disease (Simate *et al*., 2012). In addition to the pH of the water, the operation process is also dependent on temperature, dosage and type of coagulant (Saritha *et al*, 2015)

In flocculation process, polymeric materials are used to form bridges between the flocs and bind the particles together to form large aggregates (Fu and Wang, 2011 and Sher *et al*., 2013). Like coagulation, once the suspended particles are bound together into large clumps, they are subsequently removed by sedimentation, filtration and flotation. Polymeric flocculants possess some advantages over coagulants in that they have the ability to produce large, dense and compact flocs that are stronger and have good settling features compared to those produced by coagulation. Polymeric flocculants are easy to handle and readily soluble in aqueous systems. High removal efficiency can be obtained with a small amount of flocculant. Polymeric flocculants destabilize particles and colloidal matters by the compressing of electrical double layers, neutralizing the charge and subsequently forming a particle–polymer–particle bridge which in turn generates a small volume of sludge (Renault *et al*., 2009). Unlike coagulation, this process is less dependent on pH.

Notwithstanding, chemical consumption makes the cost of operation high (Kurniawan *et al*., 2006a) and cannot be effectively used for wastewater treatment unless accompanied by other treatment methods (Chang and Wang, 2007).

1.2.3 Flotation

Flotation uses bubble attachment to separate solids or dispersed liquids from a liquid phase (Fu and Wang, 2011 and Parmar and Thakur, 2013). The suspended particles are separated from the suspension of heavy metals by adhering to the surface of rising bubbles. Chemical pre-treatment is essential for effective flotation. It is preferably used instead of sedimentation after coagulation/flocculation because the time of operation is shorter than that of the latter. There are various types of flotation namely: dissolved-air, dispersed-air, vacuum air, biological and electro-flotation (Parmar and Thakur, 2013).

Dissolved-air flotation (DAF) is the most widely used type of flotation for metal removal from wastewaters (Parmar and Thakur, 2013). DAF is effective in the removal of low density particles that are capable of floating (Hami *et al*., 2007). In DAF, air is dissolved at high pressure in a saturator, followed by the formation of micro-bubbles at the release of water in the flotation tank at atmospheric pressure (Al-Shamrani *et al*., 2002). Suspended particles in the wastewater adhere to the surface of the micro-bubbles to form low density aggregates that float on the water where they can be collected as sludge (Fu and Wang, 2011).

Ion flotation technique is a process whereby a surface active ionic collector is used to transport a non-surface active ionic solute called the colligend from a bulk solution to the solution-vapour interface. The colligend in this process is usually of opposite charge to the ionic collector (Yuan *et al*., 2008).

1.2.4 Membrane filtration

The membrane filtration process is a promising technique capable of removing organic and inorganic contaminants as well as suspended solids, nitrogen, phosphorus and bacteria (Blšťáková *et al*., 2009 and Parmar and Thakur, 2013). Different types of membrane filtration process have been employed in the removal of heavy metals from wastewaters based on the size of particle that can be retained. They include ultrafiltration, nanofiltration, reverse osmosis and electrodialysis.

Ultrafiltration (UF)

The UF process uses permeable membranes to separate heavy metals, macromolecules and suspended solids from inorganic solutions based on the pore size and molecular weight of the separating compounds. Water and low molecular weight solutes are allowed to pass through the membrane while molecules with larger masses are retained (Kurniawan *et al*., 2006a and Barakat, 2011).

UF only requires a small operating space and less labour requirements. However, its use is limited by membrane fouling (Fu and Wang, 2011 and Parmar and Thakur, 2013).

Reverse osmosis (RO)

RO is a pressure induced membrane process. It uses a semi-permeable membrane and pressure forces the influent through the membrane which allows treated water to pass through while withholding the contaminants (Fu and Wang, 2011 and Parmar and Thakur, 2013). RO membranes have a dense barrier layer in the polymer matrix which aids separation (Gautum *et al*., 2014). RO has the ability to remove a large number of dissolved matters from water and is known for its high salt rejection ability which makes it favourable for desalination. RO presents a number of advantages which include high water flux rate, ability to withstand high temperature, high efficiency, chemical stability and resistance to biological attack. The major disadvantage of RO however is high energy consumption due to high pressure requirement and

high operational cost due to membrane replacement to avoid fouling (and Fu and Wang, 2011 and Parmar and Thakur, 2013).

Nanofiltration (NF)

Nanofiltration process is the stage in-between UF and RO, and as such possesses exceptional properties between the two (Al-Rashdi *et al*., 2013). Due to NF's small pore size and membrane surface charge, the membrane rejects charged solutes that are smaller than its pores alongside large solute and salts. NF unlike RO requires lower pressure and thus low operational cost (Fu and Wang, 2011). Like UF, NF is also limited by membrane fouling.

Electrodialysis (ED)

ED is another type of membrane separation technique which uses an ion-exchange membrane. In ED, metal ions are separated by passing the solution through an ion-exchange membrane with the use of an electric field. ED usually employs either a cation-exchange membrane or an anion-exchange membrane (Fu and Wang, 2011). ED has been proven to be very effective in the removal of metals like Cu and Fe and is also able to recover water from solutions in copper electrowinning processes (Cifuentes *et al*., 2009). It is however restricted due to inadequacy to efficiently remove heavy metals from inorganic effluent with high metal concentration (> 1000mg/L) (Kurniawan *et al*., 2006a).

1.2.5 Ion exchange

Ion exchange treatment technique is another method used in the removal of heavy metals from wastewaters. It is one of the most widely used treatments owing to its numerous advantages which include, high removal capability and fast reaction rates (Fu and Wang, 2011). The possibility of ion-exchange depends on the selective capacity of the exchanger for the heavy metal to be removed and the concentrations of competing ions. Low concentrations of competing ions and a high selective ability of an exchanger are required for ion exchange (Hui *et al*., 2005). Synthetic insoluble substances called resins are the most often used exchanger (Fu and Wang, 2011). Ion exchange can as well be used to recover heavy metals. Metals can be recovered either after the separation of the loaded resin by elution with appropriate reagents (Kurniawan *et al*., 2006a) or by incinerating the metal-saturated resin (Hui *et al*., 2005). The most widely used ion exchangers consists of either the sulfonic acid groups or the carboxylic acid groups which are the strongly or weakly acidic resins respectively (Fu and Wang, 2011). The physicochemical exchange that is likely to occur during the uptake of metal is represented in the following equations

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$$n\text{R–SO}_3\text{H} + \text{M}^{n+} \rightarrow (\text{RSO}_3^-)_n\,\text{M}^{n+} + n\text{H}^+ \quad (1)$$

(resin) (solution) (resin) (solution)

(Fu and Wang, 2011)

$$n\text{R–COOH} + \text{M}^{n+} \rightarrow (\text{RCOO}^-)_n\,\text{M}^{n+} + n\text{H}^+ \quad (2)$$

(resin) (solution) (resin) (solution)

(Fu and Wang, 2011)

According to Gode and Pehlivan (2006), a number of factors like temperature, initial metal concentration, contact time and pH somewhat affects the ability of ion-exchange resins to take-

up heavy metal ions. The high cost of this process generally confines its use to only the very important metals.

1.2.6 Electrochemical treatment

Electrochemical treatment of water has been introduced in the UK since 1889 but did not gain extensive and progressive utilization due to reasons ranging from the investment of a rather large capital to costly power supply (Chen, 2004). Electrochemical treatment methods have regained their attractiveness over the last two decades because of the increasing strict environmental regulations on wastewater discharge. Electrochemical treatment process for heavy metal removal involves various technologies such as electrocoagulation (EC), electro-flotation (EF), electro-oxidation and electrodeposition (Chen, 2004 and Fu and Wang, 2011).

Electrocoagulation

Electrocoagulation is a process whereby coagulants are produced in-situ by electrically breaking up either aluminium or iron ion from their respective electrodes (Chen, 2004). Production of metal ion occurs at the anode while hydrogen gas generation takes place at the cathode. The hydrogen gas assists in floating the already flocculated elements out of the water (Fu and Wang, 2011). The benefits of this technology include high removal efficiency and low cost of operation compared to other electrochemical techniques.

Electro-flotation

Electro-flotation is a separation technique that relies on hydrogen and oxygen gases produced during the passage of electric current though water (Hosny, 1996). The uniform tiny gas bubbles generated on electrode surface cleaves the pollutants and causes them to float to the surface of the water (Chen, 2004 and Fu and Wang, 2011). Unlike other flotation systems, the electro-flotation systems are petite, which makes them less expensive and easy to maintain (Hosny, 1996). In addition, because gas bubbles generated via electrolysis in electro-flotation

are tiny and uniform, larger surface area for attachment of floating particles is made available (Chen, 2004).

Electro-oxidation

Electro-oxidation of pollutants is in two major forms. It could be directly or indirectly. Direct electro-oxidation of pollutants may take place literally on anodes by producing naturally adsorbed "active oxygen" which brings about the total combustion of organic compounds or chemisorbed "active oxygen" that takes part in the creation of selective oxidation compounds. This method is commonly referred to as anodic oxidation (Chen, 2004). This process is compact and tends not to produce sludge as it neither requires the addition of large volume of chemicals to wastewater nor oxygen to cathodes.

Indirect electro-oxidation can be accomplished through various means. It can be achieved by using anodically created chlorine and hypochlorite to break down pollutants. This method can successfully oxidize several organic and inorganic contaminants at high chloride concentration (Chen, 2004). However, the process is limited by the likely formation of chlorinated organic compounds intermediates or resultant products (Naumczyk *et al*., 1996). In addition, in the case of low chlorine content in raw waste water, more salt will definitely be required to boost the efficiency of the process (Chen, 2004). Indirect electro-oxidation can also be carried out by using hydrogen peroxide (H_2O_2) produced electrochemically to breakdown pollutants (Brillas *et al*., 1998 and Chen, 2004). The cathode in this process consist of carbon-polytetrefluorethylene (PTFE) fed with oxygen while the anode is Pb/PbO_2 or a Pt anode. The oxygen fed cathode electrochemically generates the H_2O_2 used for breakdown in the following reaction (Brillas *et al*., 1998).

$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2$$

Electrodeposition

Electrodeposition is generally known for its use in the recovery of heavy metals from wastewater (Fu and Wang, 2011). This method was reported to be suitable for recovering heavy metals under desirable conditions (Oztekin and Yazicigil, 2006 and Issabayeva *et al*., 2006). This technique is regarded as a "clean" method (lacking long-lasting sediments) for the separation of heavy metals (Issabayeva *et al*., 2006). According to Chang *et al*., (2009), the combination of electrodeposition and ultrasound efficiently removed copper (95.6%) and 84% COD from wastewater.

1.3 Adsorption

Adsorption process is presently acknowledged as one of the alternative treatment methods for heavy metal wastewater (Bhattacharyya and Gupta, 2008 and Fu and Wang, 2011). "Adsorption is a mass transfer process" (Kurniawan *et al*., 2006a) which when carried out in conjunction with appropriate desorption methods resolves the issue of sludge disposal (Gupta *et al*., 2010). The process involves the attachment of a substance onto a solid by either physical and/or chemical interactions. The substance (pollutant) is usually transferred from the bulk solution (liquid phase) to the surface of the solid (sorbent) onto which the substance binds (Barakat, 2011 and Kurniawan *et al*., 2006a). Adsorption is a simple, efficient and flexible method for heavy metal removal in wastewater (Bhattacharyya and Gupta, 2008). Its advantages include low capital cost, suitability at very low concentrations, little sludge production, ease of operation and sorbent regeneration by suitable desorption process as a result of possible process reversal (Bhattacharyya and Gupta, 2008 and Fu and Wang, 2011). Likewise, sorbents can be incorporated into waste treatment systems with ease and it is applicable for both batch and continuous process (Pyrzńska and Bystrzejewski, 2010). An adsorbent is referred to as "good" if it has a porous structure giving rise to large surface area and exhibits fast adsorption kinetics (Gupta *et al*., 2009). According to Gupta and Suhas

(2009), adsorption can be of two types. The first one – 'physiosorption' or physical adsorption is the type of adsorption that occurs naturally by physical attraction between the solid surface and the adsorbed molecules while the type in which attraction is facilitated by forces through chemical bonding is called chemisorption.

1.3.1 Activated carbon adsorption

Activated carbon (AC) is the most commonly used adsorbent in the treatment of heavy metal wastewater (Babel and Kurniawan, 2003 and Fu and Wang, 2011). Adsorption using activated carbon has been used in the removal of various heavy metals due to its large micropore and mesopore volumes (which results in its large surface area), surface reactivity and high capacity for adsorption (Fu and Wang, 2011 and Kurniawan *et al*., 2006a). Adsorption absolutely depends on the type of activated carbon, its surface area and permeability (Bhole and Ramteke, 2011). Activated carbon usually comprises of graphene pieces which are randomly alternated with hetero-atoms. The features of the resulting activated carbon are dependent on the material and the activation technique used in making it (Pyrznska and Bystrzejewski, 2010). There are two types of activation method – physical activation and chemical activation. Either of these methods is used in the preparation of AC from materials like lignite, coal, coconut shells and wood which are referred to as precursors (Gupta *et al*., 2009). In physical activation, the precursor is normally made to undergo carbonization first, then activation to form AC, or in some cases either of the two processes is employed. In chemical activation, precursors are usually impregnated with chemicals like such as H_3PO_4, KOH, or NaOH before subjection to heat at 450 to 900^{o}C to activate the carbons (Gupta *et al*., 2009). Despite the wide application of AC, investigations on production and/or exploration of alternative adsorbents are being made since the depletion in the source of commercial coal based AC has led to increase in the cost of operating the process (Babel and Kurniawan, 2003 and Fu and Wang, 2011). Due to the high cost of commercially available ACs, researchers have been searching for alternative

activated carbon from inexpensive sources for economical wastewater treatment (Fu and Wang, 2011). Anoop Krishnan *et al*. (2011) reported that an activated carbon obtained from sugarcane bagasse pith (SBP-AC) was effectively used in the removal of Ni (II) from aqueous solution and was found to be more effective than commercial activated carbon. Anirudhan and Sreekumari (2011) showed that activated carbon produced by steam activation of coconut buttons can be used as a potential adsorbent in the removal of Pb (II), Hg (II) and Cu (II) ions from aqueous solutions. Activated carbon prepared by pyrolysis of potato peel impregnated with zinc chloride solution is applicable for the removal of copper ions from industrial effluents (Moreno-Piraján and Giraldo, 2011). The cost of preparing these activated carbons are said to be low since raw materials obtained from agricultural wastes are readily available and in abundance.

1.3.2 Low cost adsorbents

Even though AC has been widely used as an adsorbent in wastewater treatment operations, it is a rather expensive treatment method (Fu and Wang, 2011). Besides, activated carbon involves the use of complexing substances for efficient removal of inorganic compounds including heavy metals (Babel and Kurniawan, 2003) and its regeneration also requires the use of costly chemicals which makes it difficult and thus restricts its application (Ali, 2010). According to Bailey *et al*., (1999), a cheap material that is abundant in nature, needs little processing and is a derivative of waste material from waste industry can be referred to as an adsorbent.

As a result of the difficulties encountered with AC, a number of investigations (Gupta and Ali, 2006 and Pollard *et al*., 1992) have been carried out for the purpose of developing and producing new and inexpensive adsorbents that will replace the expensive activated carbon for the removal of heavy metals from wastewaters (Ali, 2010). Several non-conventional sorbents such as agricultural waste, industrial by-products, natural substances, biomass and biopolymers have been suggested and studied as adsorbents with metal binding capability for the treatment

of wastewater (Fu and Wang, 2011 and Sancey *et al*., 2011). They include lignin, fly ash, blast furnace sludge, rice hulls, fruit stones, sawdust, waste slurry, red mud, sugar beet pulp, peat moss, coffee husks, battery industry waste, grape stalk wastes, areca waste, sea nodule residue, waste biogas residual slurry, tea factory waste, chitin and chitosan, zeolite, petroleum waste, clays and dead biomass (Ahmaruzzaman, 2011, Ali, 2010, Gupta *et al*., 2009 and Sud *et al*., 2008).

1.3.3 Industrial by-products

Industrial by-products obtained as a result of various industrial activities can be used as adsorbents in the removal of heavy metals from wastewaters laden with heavy metals (Kurniawan *et al*., 2006b). They are cheap and readily available in abundance. They include fly ash, blast furnace slag, waste iron, waste slurry, lignin, iron (III) hydroxide, and red mud (Ahmaruzzaman, 2011, Babel and Kurniawan, 2003 and Kurniawan *et al*., 2006b). These adsorbents can be altered chemically to improve their removal efficiency (Kurniawan *et al*., 2006b).

Fly ash

Fly ash is an inorganic by-product largely comprising of 40–50% (w/w) silica (SiO_2), 20–35% (w/w) alumina (Al_2O_3) and 5–12% (w/w) iron oxide (Fe_2O_3). It also comprises of 12–30% (w/w) carbon, magnetite and unburnt residue (Ahmaruzzaman, 2010, Ahmaruzzaman, 2011 and Ali, 2010). It has a specific gravity that ranges between 2.1 and 3.0 and a specific surface area ranging from 170 to 1000 m^2/kg. The amount of unburnt carbon present in a specific fly ash determines the colour of the ash which alternates between tan, grey and black (Ahmaruzzaman, 2010). Fly ash can be produced in large quantity as a result of power generation from coal firing in thermal power plants (Gupta *et al*., 2009) and are usually referred to as coal fly ash (CFA). Fly ash can also be obtained from sugar industries and brick kilns as waste and/or by-products (Ali, 2010). Fly ash is highly available in large quantity at

little or no cost, and its disposal has come to be a crucial environmental issue in several places (Ahmaruzzaman, 2010 and Gupta *et al*., 2009). Due to this fact, researchers have investigated the use of fly ash as an adsorbent in the treatment of heavy metals contaminated wastewater (Ahmaruzzaman, 2010, Ahmaruzzaman, 2011, Ali, 2010 and Gupta *et al*., 2009). The physicochemical characteristics of fly ash which includes porosity, bulk density, particle size, surface area and ability to retain water deems it fit for use as an adsorbent. Its alkalinity also implies that it is a good neutraliser. However, its adsorption performance is dependent to a large extent on the origin (i.e. the type of coal from which it was obtained) of the fly ash and chemical treatment (Ahmaruzzaman, 2011). Basically, fly ash can result from four different sources namely: lignite, anthracite, sub-bituminous and bituminous. Bituminous coal fly ash consists of silica, alumina, iron oxide and calcium, with varying amounts of carbon, as measured by the loss on ignition. Unlike bituminous coal fly ash, fly ash obtained from lignite and sub-bituminous coal is made up of higher concentrations of calcium and magnesium oxide and lower concentrations of silica, iron oxide and carbon (Ahmaruzzaman, 2010). Anthracite coal fly ashes are available only in little quantities as extremely little anthracite coal is burned in utility boilers. Fly ash produced from lignite and sub-bituminous coals (low ranking coals) have a high percentage of lime, high amount of alkalis and between 50% and 70% $SiO_2 + Al_2O_3 + Fe_2O_3$ are characterised as class C fly ash while those generated from higher rank coals, bituminous coals or anthracites which have a low lime content, lower alkalis and >70% $SiO_2 + Al_2O_3 + Fe_2O_3$ are termed class F fly ash (Ahmaruzzaman, 2010). The study of Cetin and Pehlivan (2007) established the potential use of fly ash from coal-fired power plant for the removal of Zn (II) and Ni (II) from aqueous solutions. The efficiency of fly ash as an adsorbent got better with increasing calcium (CaO) content and was found to be as effective as activated carbon. According to Weng and Huang (2004), Zinc adsorption onto fly ash was up to 99% at a specific pH. The study showed that the adsorption was good at lower ionic strength, higher pH, and higher temperature as adsorption rate increased with increase in pH

and solid concentration. Sharma *et al*. (2008) also investigated the potential of coal fly ash as an adsorbent for chromium removal from wastewaters and aqueous solution and found out that chromium removal was controlled by intraparticle diffusion which had a coefficient value of 2.25×10^{-11} cm^2/s at 298 K. This value implies a rapid transfer of Cr onto the surface of the adsorbent. The removal process depended on the pH of the solution to a large extent with highest removal of 89.12% at pH 2.5.

There is also what is called "biomass fly ash" which can be produced in large quantities from the utilization of biomass as fuel (e.g. rice straw, wheat straw, sugar cane straw and wood) (Ahmaruzzaman, 2010 and Thy *et al*., 2006). The components of biomass fly ashes really differ based on the type of biomass, type of soil and harvesting (Thy *et al*., 2006). Generally, the major inorganic elements responsible for the formation of ash in biomass fuels are calcium, potassium, sodium, silicon and phosphorus. The elemental composition of individual biomass fly ash thus depends on the inorganic components of the precursor (Ahmaruzzaman, 2010). Some biomass fuels such as rice husk are high in silicon whereas some like wood contain high alkali metal. Bagasse fly ash produced as a waste from the sugar industry and which is composed of 60.5% SiO, 15.4% Al_2O_3, 2.90% CaO, 4.90% Fe_2O_3 and 0.81% MgO was employed for the removal of copper and zinc from wastewater under suitable condition with removal capacity of 93-98% (Gupta and Ali, 2000). Gupta *et al*. (2003) used bagasse fly ash in the removal of cadmium and nickel from wastewater with as much as 90% removal.

Blast furnace slag

Blast furnace slag (BFS) is a waste and/or by-product generated in large quantity from steel production in steel plants (Ahmaruzzaman, 2011, Ali, 2010 and Kurniawan *et al*., 2006b). It is usually employed as filler or in the manufacture of slag cement. Lately, as a result of its heavy metals adsorption capability, it has been developed into an inexpensive adsorbent for the removal of heavy metal ions from aqueous solutions (Ahmaruzzaman, 2011 and Ali, 2010).

Dimitrova and Mehanjiev (2000) employed BFS for Cu, Zn and Ni ions removal. It was observed that BFS adsorbs metal ions based on two mechanisms (i) ion exchange and (ii) exchange and replacement of calcium ions from the slag by the metal ions in the solutions. Adsorption of metal ions occurred alongside partial solubility and hydrolysis of slag minerals such as calcium silicates and aluminosilicates. Based on the work of Feng *et al*. (2004), metallurgical by-product slags which include BFS are suitable adsorbents for the removal of heavy metals from wastewater. Nehrenheim and Gustafsson (2008) also reported the use of blast furnace slag as a sorbent in the removal of Cu (II), Ni (II), Zn (II), Pb (II) and Cr (III) metal ions. Contact time was the most important factor regulating adsorption. Blast furnace slag, even though fast in binding to metals, was more efficient in the removal of metal ions at increased metal concentration than at rather low metal concentrations. The data was based on a pseudo-second-order kinetic model. The sorption capacity of BFS is dependent on pH of the aqueous solution since the sorption capability increases intensely with an increase in the pH of the solution (Kim *et al*., 2008).

Red mud

Red mud is yet another industrial by-product or waste material that is available in great quantity (Ahmaruzzaman, 2011). Red mud is generated when bauxite ore is exposed to caustic leaching (a process called Bayer) while producing alumina (Ahmaruzzaman, 2011 and Ali, 2010). A Bayer process usually produces about 1-2 tons of red mud per every ton of alumina produced. Red mud is reddish-brown in colour with a superfine particle size distribution (Nadaroglu *et al*., 2010). The major components of red mud which include silica, aluminium, iron, calcium and titanium oxides and hydroxides are responsible for its high surface reactivity and porosity (Ahmaruzzaman, 2011, Ali, 2010, Bhatnagar *et al*., 2011 and Nadaroglu *et al*., 2010). Due to these elements, several studies have been carried out on the utilization of red mud for the removal of heavy metals in wastewater treatment and it is proven to be a potential adsorbent in the removal of heavy metals (Bhatnagar *et al*., 2011). Red mud has been

investigated by Gupta *et al.* (2001) for its use as a low-cost adsorbent in the removal of lead and chromium from aqueous solution. The adsorption process which occurred through particle diffusion showed good adsorption ability and the thermodynamic parameters reflect the feasibility of the process. The study of Nadaroglu *et al*. (2010) also showed that red mud is a suitable adsorbent for removal of copper ions from aqueous solutions laden with heavy metals. Adsorption rate increased with a rise in temperature. An increase in temperature resulted in an increase in mobility of the metal ions and a fall in the retarding forces acting on the diffusing ions. Maximum adsorption was achieved at 30 ºC which is the temperature of the solution. Granular red mud was shown to be an effective adsorbent with high cadmium ion adsorption capability at low concentrations in the report of Zhu *et al*. (2007).

1.3.4 Agricultural Wastes

In addition to industrial by-products, agricultural waste is another source of inexpensive adsorbents (Kurniawan *et al*., 2006b). Like industrial by-products, they are cheap, readily available and in abundance. Their exceptional chemical composition makes them environmental friendly and they are also renewable (Sud *et al*., 2008). Reports (Cimino *et al*., 2000, Hashem *et al*., 2006, Montanher *et al*., 2005 and Sud *et al*., 2008) have shown that a number of studies have been carried out on the utilisation of agricultural wastes for heavy metal removal. Some of the employed agricultural wastes include rice bran, cotton seed hulls, rice husk, wheat bran, wheat husk, banana peels, saw dust of various plants, bark of the trees, orange peels, groundnut shells, sugarcane bagasse, coconut shells, grapes stalks, hazelnut shells, black gram husk, walnut shells, waste tea leaves, water hyacinth, *Cassia fistula* leaves, sunflower stalks, maize corn cob, sugarcane bagasse, coffee beans, soybean hulls, sugar beet pulp, etc. (Sud *et al*., 2008).

Rice husk/hull

Rice husk is an agricultural waste product generated in large quantity as a result of rice production at rice mills (Kumar and Bandyopadhyay, 2006). Its disposal poses problem to the environment because it generates CO_2 and other pollutants when burnt in-situ. Hence, its use as an adsorbent will protect the environment from pollution. Rice husk is composed of 32.24% cellulose, 21.34% hemicellulose, 21.44% lignin, 15.05% mineral ash and a high percentage of silica (approx. 96.34%) in its mineral ash (Wan Ngah and Hanafiah, 2008). About 0.23 tons of rice husk is generated per every ton of rice processed (Kumar and Bandyopadhyay, 2006). Rice husk possesses properties such as good chemical stability, a granular structure, high mechanical strength and non-solubility in water which makes it a potential adsorbent for removing heavy metals from aqueous solution (Wan Ngah and Hanafiah, 2008). Much attention has been dedicated on the investigation and utilization of modified and/or unmodified rice husk as adsorbent for the removal of heavy metals from polluted waters in recent years. Rice husks can be chemically modified or pre-treated to eliminate lignin and hemicellulose, increase its surface area or porosity and decrease cellulose crystallinity for better performance. Compounds like hydrochloric acid, sodium hydroxide, sodium carbonate, epichlorohydrin and tartaric acid have been used in the chemical modification of rice husk (Wan Ngah and Hanafiah, 2008). Kumar and Bandyopadhyay (2006) investigated the adsorption capability of raw rice husk (RRH), epichlorohydrin treated rice husk (ERH), NaOH treated rice husk (NRH), sodium bicarbonate treated rice husk (NCRH) for the removal of cadmium from aqueous solution and found that equilibrium time decreased from 10 h in RRH to 2, 4 and 1 h while sorption capacity increased from 8.58 mg/g for RRH to 11.12, 20.24, 16.18 mg/g for ERH, NRH and NCRH respectively. Mohan and Sreelakshmi (2008) reported that both raw rice husk (RRH) and phosphate treated rice husk (PRH) were efficient in the removal of heavy metals like manganese, copper, lead and zinc in continuous mode using fixed bed column although PRH exhibited higher adsorption capacity and adsorption rate constant. The study of

Bansal *et al*. (2009) also presented that pre-boiled rice husk (BRH) and formaldehyde treated rice husk (FRH) can be used as adsorbents for the removal of Cr(VI) from wastewaters with removal efficiency of 71.0% and 76.5% for BRH and FRH respectively.

Fruit peels

Peels of different fruits are important agricultural wastes. Their use as adsorbents is presently getting wide attention since they are available in abundance and are relatively cheap due to their high fixed carbon content and presence of porous structure (Bhatnagar *et al*., 2010). Since fruit peels are a major agricultural waste that constitutes nuisance in different parts of the world, they have been investigated as adsorbents for the removal of various types of pollutants especially metal ions from wastewater (Anwar *et al*., 2010). Various fruits peels which include orange, banana, lemon, cassava and mango have been used as adsorbents for the removal of heavy metals from wastewater. Anwar *et al*. (2010) studied the adsorption of Pb (II) and Cd (II) on banana peels in batch mode. The results obtained showed that fruit wastes such as banana peels can be used in the removal of heavy metals from wastewaters and industrial effluents. Also, the study of Bhatnagar *et al*. (2010) showed that peels of lemon can be used as an efficient adsorbent for the removal of cobalt from aqueous solutions. The adsorption capacity of lemon peel for cobalt at 25^{o}C was 22mg/g. The process was spontaneous and adsorption data better fitted the pseudo-second-order kinetic model. Li *et al*. (2008) studied the adsorption of Co (II), Ni (II), Zn (II) and Cd (II) on raw and chemically modifies orange peels. Orange peels were initially washed with 20% iso-propyl alcohol, and thereafter saponified with NaOH. After saponification with NaOH, the peels were chemically modified with oxalic acid (SOA), phosphoric acid (SPA) and citric acid (SCA). The maximum adsorption capacities of Ni (II), Co (II), Zn (II) and Cd (II) for SPA, SPA, SCA and SOA increased by 95, 178, 60 and 130% respectively when compared to raw orange peel. The work of Iqbal *et al*. (2009) assessed the potentiality of mango peels as adsorbent for the removal and recovery of cadmium and lead ions from aqueous solution. Mango peel was established to be an effective and low-

cost adsorbent for the removal and recovery of cadmium and lead ions from aqueous solution with maximum adsorption capacity of 68.92 mg/g and 99.05 mg/g respectively. Adsorption data fitted pseudo-second order and Langmuir adsorption isotherm models. Metal ions adsorption depended on pH, sorbate and sorbent concentrations and sorbate – sorbent contact time.

Sugarcane bagasse

Sugarcane bagasse is an abundant agricultural waste with metal-binding capability usually obtained from sugarcane industries. Sugarcane bagasse comprises of three biological polymers namely cellulose (50%), polyoses (27%) and lignin (23%) which makes it very rich in hydroxyl and phenolic groups. These groups usually require chemical modification to form compounds with new properties such as increased sorption capacity (Júnior *et al*., 2007). Homagai *et al*. (2010) investigated the use of charred xanthated sugarcane bagasse (CXSB) as an adsorbent for the removal of Cd (II), Pb (II), Ni (II), Zn (II) and Cu (II) from aqueous solutions. The material showed high adsorption capacity compared to other chemically modified sugarcane bagasse. The report of Júnior *et al*. (2009) also stated the use of succinic anhydride modified sugarcane bagasse for the removal of copper, cadmium and lead from aqueous solutions. Furthermore, the study of Sanchez and Esposito (2011) described the modification of sugarcane bagasse with thiophosphoryl chloride to form a new material called SCB-F. The new material was used in the removal of cadmium from aqueous solution. It showed increased cadmium adsorption capacity and maximum cadmium adsorption of 74 mg/g which is more than 60 times higher when compared with of unmodified sugarcane bagasse. Chemical modification of sugarcane bagasse with succinic anhydride for the removal of Zn (II) ions from electroplating wastewater is described in the work of Pereira *et al*. (2009). Carboxylic acid functions were introduced into the new material via succinic anhydride modification and the material was further characterized by infrared spectroscopy. Adsorption

capacities were found to be higher (145 mg/g) in single metal aqueous solution than in industrial wastewater (55 mg/g).

Sawdust of various plants

Sawdust is another abundant agricultural waste usually obtained as a by-product from wood industry at little cost (Wan Ngah and Hanafiah, 2008). Its constituents include lignin, cellulose, hemicellulose and polyphenolic groups which uses different mechanisms to bind heavy metal ions. Naiya *et al*. (2009) investigated the capability of sawdust as a cost-effective adsorbent for the removal of zinc and cadmium ion from aqueous solution. Adsorption of heavy metals was found to be dependent on pH and the optimum pH for zinc and cadmium ions were found to be 5 and 6 respectively. Adsorption kinetics data best fitted into pseudo-second order model while equilibrium adsorption data followed Langmuir isotherm model for zinc and Freundlich isotherm model for cadmium adsorption. An increase in the quantity of adsorbent increased the number of adsorption sites, thereby increasing metal ion adsorption. Meena *et al*. (2008) evaluated the possibility of using NaOH and H_2SO_4 treated sawdust for the removal of heavy metals like Cr (VI), Hg (II), Cu (II) and Pb (II) from aqueous solution. Removal efficiency was determined by parameters such as contact time, temperature, pH, concentration and dosage of adsorbent. It was discovered that the treated sawdust exhibited almost 100% removal of metal ions under optimized conditions (1 g/100ml of adsorbent for aqueous solution having 3 mg/L metal ions for 48 hours). Adsorption isotherms followed both Langmuir and Freundlich isotherm models. The work of Rafatullah *et al*. (2009) also assessed the ability of sawdust obtained from meranti tree to adsorb Cu (II), Cr (III), Ni (II) and Pb (II) ions from aqueous solutions. Meranti sawdust was found to be a suitable low cost adsorbent for heavy metals removal from aqueous solution. The removal of these metal ions was dependent on temperature, metal ions concentration and adsorbent dose. Increases in concentration and dosage of adsorbent increased the amount of Cu (II), Cr (III), Ni (II) and Pb (II) ions adsorbed and the rate of adsorption increased with increases in temperature. Maximum adsorption was

achieved at pH 6 and adsorption equilibrium was reached at 120 min contact time. Adsorption kinetics and isotherms followed pseudo-second-order and Langmuir isotherm models respectively (Rafatullah *et al*., 2009).

1.3.5 Natural materials

Locally obtainable naturally materials can also be used as low cost adsorbents. Natural materials like zeolite, chitin and chitosan, wood, coal, biomass, alginate and clay have been utilised in the removal of heavy metals from industrial effluents (Babel and Kurniawan, 2003, Gupta *et al*., 2009 and Kurniawan *et al*., 2006b).

Zeolite

Zeolites are hydrated aluminosilicate minerals that occur naturally (Ibrahim *et al*., 2010). Natural zeolites are usually formed as a result of alteration of glass-rich volcanic rocks with either seawater or fresh water in Playa lakes and are part of the class of minerals called 'tectosilicates' (Ibrahim *et al*., 2010) Clinoptilolite, mordenite, laumontite, chabazite and analcime and are amongst the well-known natural zeolites with clinoptilolite being the most abundant while some other forms such as mazzite offretite, barrerite and paulingite are uncommon (Wang and Peng, 2010). Structurally, zeolites consist of three-dimensional frameworks of SiO_4 and AlO_4 tetrahedra linked by shared oxygen atoms. The centre of the tetrahedron of four oxygen atoms is occupied by the aluminium ion and a net negative charge is generated as a result of the isomorphic replacement of Si^{4+} by Al^{3+} in the mineral lattice (Jamil *et al*., 2010 and Rosales *et al*., 2012).

They are recognized excellent adsorbents that can adsorb and have exchangeable ions such as Na, Ca and K that have strong attraction for metal cations in solutions as a result of the positive charges in their framework (Han *et al*., 2009, Ibrahim *et al*., 2010, Jamil *et al*., 2010 and Rosales *et al*., 2012). These exchangeable ions are specifically appropriate for heavy metals removal from wastewaters due to their harmless nature (Ibrahim *et al*., 2010). The chemical

composition and cation-exchange capacity of natural zeolites differs due to the environment in which they are formed (Wang and Peng, 2010). Because there are different zeolite species, heavy metals sorption studies carried out under the same test conditions usually results in different selectivity choices and sorption capacities (Ziyath *et al*., 2011). Therefore, the key features of zeolites that control their performance in the removal of heavy metals are ion exchange capability, high surface area, pore size and aluminium content or silicon to aluminium ratio (Ibrahim *et al*., 2010 and Ziyath *et al*., 2011).

Chitosan

Chitosan, a kind of natural poly-aminosaccharide is derived from the deacetylation of chitin, the world's second most abundant polysaccharide in nature. Chitin is made up of mostly unbranched chains of β-(1→4)-2-acetoamido-2-deoxy-d-glucose and is usually from the exoskeleton of crustaceans such as prawns, crabs, shrimps, lobsters and insects (Gerente *et al*., 2007 and Wan Ngah *et al*., 2011). Chitosan is commonly used in the treatment of polluted water and as chelating agent in adsorbing heavy metals from industrial wastewater due to its low toxic, biocompatible and biodegradable features (Liu *et al*., 2012). Benavente *et al*. (2011) investigated the use of chitosan produced from waste of shrimp shell in the removal of Cu (II), Hg (II), Pb (II) and Zn (II) from gold ore tailing solutions. Results showed that chitosan was efficient in the removal of all the metal ions above 70%.

1.4 Biosorption

Biological methods based on bioremediation techniques which include accumulation and/or biosorption of heavy metals by microorganisms have been developed and are more effective, cheaper and less invasive than traditional methods (Witek-Krowiak *et al*., 2011).

Biosorption is a method that can be used to remove contaminants from wastewaters, particularly those contaminants that are difficult to biodegrade such as metals and dyes (Vijayaraghavan and Yun, 2008). A number of biological substances like bacteria, fungi, algae,

and industrial and agricultural wastes have been reported to bind these pollutants. In recent years, biological methods of metal removal based on bioremediation techniques such as the active uptake of metals by microorganisms (bioaccumulation) and/or passive uptake by microorganisms and/or their products (biosorption) have emerged (Hemambika *et al*., 2011).

Biosorption is somewhat a new development that has shown to be effective in the removal of heavy metals from aqueous solution and is fast becoming a promising substitute to conventional techniques used in the removal and/or recovery of heavy metals from wastewater (Demirbas, 2008). Biosorption is said to be more suitable than bioaccumulation because while the former uses either live or dead microorganisms or their product for removal, the latter which uses live organisms usually require the addition of nutrients and thus increase biological oxygen demand (BOD) or chemical oxygen demand (COD) in the effluent (Hemambika *et al*., 2011 and Yan and Viraraghavan, 2003). A biosorbent can be considered low cost if it requires little processing, is abundant in nature, or is a by-product or waste material from another industry (Hlihor and Gavrilescu, 2009)

The biosorption process also presents various preferences over physicochemical methods such as reduction of chemical or biological sludge to the barest minimum, low cost of treatment, high efficiency and biosorbent regeneration for possible reclamation. Two phases - solid and liquid phase are involved in the biosorption process. The solid phase which is normally a biological substance is the biosorbent while the liquid phase is a solvent usually water containing the element and/or compound to be adsorbed (sorbate). The biosorbent has a higher affinity for the sorbate and as such attracts and binds the latter (Alluri *et al*., 2007). Complex mechanisms such as ion-exchange, complexation, electrostatic attraction and microprecipitation are said to be responsible for metal ion binding during biosorption (Volesky and Holan, 1995 and Wang and Chen, 2006). Biosorption is capable of being reversed and is normally a fast and cell viability independent process. Recovery allows metal recycling, leading to energy savings and material conservation (Ferraz *et al*., 2004). These bio-sorbents

are capable of binding and removing metal ions in dilute complex solutions efficiently and quickly which makes them acceptable for treating low heavy metal ion concentrations in high volume wastewaters (Wang and Chen, 2009 and Yan and Viraraghavan, 2003). Recently, bioremediation using biopolymers derived from microorganisms has been emerging as an alternative secondary treatment for the removal of these metals from aqueous systems (Inbaraj *et al*., 2009). Biopolymers are considered to be capable of sorbing heavy metals. A considerable amount of by-products which can be used in biosorption of heavy metals is generated from the synthesis of products like antibiotics, enzymes and organic acids in fermentation processes by microorganisms. In addition, various microorganisms have been reported to produce biopolymers (such as polysaccharides, peptidoglycans, poly-amino acids, lipopolysaccharides and water-soluble amphipathic exopolysaccharides) with metal-binding properties. One of such polymer that may be used for this purpose is poly-γ-glutamic acid and it has been reported by Inbaraj *et al*. (2009) that γ-PGA was effective in binding several metal ions nickel Ni^{2+}, copper Cu^{2+}, Manganese Mn^{2+}, Aluminium Al^{3+} , Cadmium Cd^{2+}, Chromium Cr^{3+} and Mercury Hg^{2+}. γ-PGA produced from *Bacillus licheniformis* ATCC 9945 was shown to have an excellent copper adsorption efficiency (Mark *et al*., 2006). γ-PGA has also been recognised as an exceptional bioflocculant due to its high flocculating ability for organic and inorganic compounds and because it is biodegradable and its degradation intermediates does not pose hazard to the environment (Bajaj and Singhal, 2011a) unlike those of organic synthetic polymer flocculants that are not readily biodegradable and at the same time some of their degraded monomers which includes acrylamide are neuro-toxic and carcinogenic (Shih and Van, 2001).

2.0 INTRODUCTION TO POLY-γ-GLUTAMIC ACID

2.1 Introduction

Polyglutamic acid (PGA) is a biodegradable, non-immunogenic and unusual anionic homopolyamide that is made of D and/or L glutamic acid units (Shih & Van, 2001).

PGA can be differentiated into 2 isoforms - α-PGA and γ-PGA depending on the attachment of the carboxy group (α and γ respectively). α-PGA is synthesized chemically by nucleophile initiated polymerization of the γ-protected N-carboxyanhydride of L-glutamic acid. Glutamic acid which is the structural monomer of poly-glutamic acid has three functional groups - α-amino acid (α-NH_2), α-carboxylic acid (α-COOH) and γ-carboxylic acid (γ-COOH) groups that react chemically in the manner α-NH_2 > α-COOH > γ-COOH. α-PGA can only be produced by recombinant technology since its microbial production is difficult (Buescher & Margaritis, 2007). There is usually a build up between the α-NH_2 and α-COOH groups in glutamic acid following its chemical polymerisation leading to the formation of an (alpha) α-peptide bond which finally results to α-polyglutamic acid (α-PGA) (**Fig. 1a**) (Buescher and Margaritis, 2007 and Ho *et al*., 2006), however in the biological synthesis using the submerged fermentation approach, enzymatic racemization occurs thereby converting a large amount of L-glutamic acid into D-glutamic acid. The less reactive α-NH_2 and γ–COOH groups come together to form a (gamma) γ-peptide bond, through which both the L-glutamic acids and the D-glutamic acids are co-polymerized to form a final product poly-γ-glutamic acid (**Fig. 1b**) (Buescher and Margaritis, 2007 and Ho *et al*., 2006).

(a) **(b)**

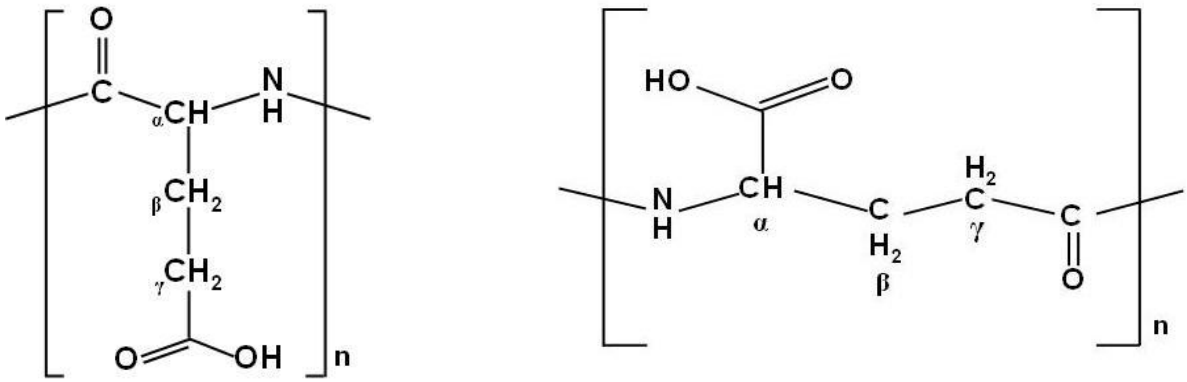


Figure 1: (a) Structure of alpha-polyglutamic acid (α-PGA) where glutamic acid residues are connected via amide linkages between α-amino and α-carboxylic acid groups (b) Structure of poly-gamma-glutamic acid (γ-PGA) glutamic acid residues are connected through amide linkages between α-amino and γ-carboxylic acid groups. Source: Ogunleye *et al*. (2015)

γ-PGA has been produced extensively using bacteria, especially those of *Bacillus* sp. It can either be composed of only L-glutamic acid residues (γ-L-PGA), only D glutamic acid residues (γ-D-PGA) or both L and D glutamic acid residues (γ-PGA). γ-PGA is different from other proteins, because inside the cell, glutamate is polymerized via the γ-amide linkages, and thus is synthesized in a ribosome independent manner (Akagi *et al*., 2007 & Bodnár *et al*., 2008). Hence, substances that inhibit translation of proteins, such as chloramphenicol, have no effect on the production of γ-PGA. Due to the γ-linkage of its component glutamate residues, γ-PGA is resistant to proteases since proteases cleave α – amino linkages (Candela & Fouet, 2006). γ-PGA can exist either in the water insoluble free acid form or as its salt with a variety of cations (Na^+, Mg^{2+}, K^+, NH_4^+ or Ca^{2+}) which is completely soluble.

γ-PGA was first discovered by Ivonovics and co-workers when a capsule of *Bacillus anthracis* was released into the medium upon autoclaving (Shih & Van, 2001). Another naturally occurring source of γ-PGA is the mucilage of natto (fermented soybeans – a traditional Japanese food) which contains a mixture of γ-PGA and fructan produced by *Bacillus subtilis* Sawamura (Candela & Fouet, 2006 and Shih & Van, 2001). γ-PGA is

produced mostly by Gram-positive bacteria which includes the *Bacillus* genus. It has also been reported that at least one Gram-negative bacterium - *Fusobacterium nucleatum*, some archaea and eukaryotes have the ability to produce γ-PGA (Candela *et al*., 2009, Hezayen *et al*., 2001 and Weber, 1990). γ-PGA has also been found in neurons of mice where it was covalently linked to tubulin (Edde *et al*., 1990). Efforts have been made to insert the genes responsible for γ-PGA production into *E. coli* and plants such as tobacco to gain more knowledge regarding the molecular mechanism for γ-PGA production (Ashiuchi *et al*., 1999a & Tarui *et al*., 2005) and resulted in the identification of *pgsB, pgsC* and *pgsA* genes as essential for its production. Recently, Cao and colleagues also cloned and co-expressed the *pgsBCA* genes and glutamate racemase gene (*racE/glr*) into *E. coli* (Cao *et al*., 2013) and reported that the engineered *E. coli* strains had the capability of synthesizing γ-PGA with excellent D-glutamate content due to *racE* integration.

2.2 Function of poly-γ-glutamic acid

The function of γ-PGA depends on the organism producing it and the environment the organism inhabits. The function also depends on whether the γ-PGA is peptidoglycan bound or released. If the γ-PGA is peptidoglycan bound it may help confer virulence or it can act as a source of glutamate in starvation conditions (Kimura *et al*., 2004 & Kocianova *et al*., 2005) whereas if γ-PGA is released into the environment, it can help the organism survive in adverse conditions (McLean *et al*., 1990).

It has been shown that the capsules of virulent strains of *B. anthracis* contain solely γ-D-PGA (Tomcsik & Szongott, 1933) and that the D enantiomer in *B. anthracis* capsule makes the bacterium non-immunogenic (Zwartouw & Smith, 1956). The γ-PGA protects the bacterial cells against phage infections and also prevents antibodies from gaining access to the bacterium (Mesnage *et al*., 1998). *Staphylococcus epidermidis* also synthesizes surface associated γ-PGA (Kocianova *et al*., 2005) which protects it against antimicrobial peptides.

In both cases γ-PGA helps the pathogenic bacteria to escape phagocytosis, hence contributing to virulence. In some cases, γ-PGA also acts as a source of glutamate when bacteria are starved in the late stationary phase (Kimura *et al*., 2004).

Unlike *B. anthracis*, certain soil bacteria release γ-PGA into the environment for the sequestration of toxic metal ions, increasing their resistance to adverse environments (McLean *et al*., 1990). Organisms such as *Planococcus halophilus*, *Sporosarcina halophila* and *Natrialba aegyptiaca* particularly use γ-PGA to decrease high local salt concentrations which helps them to survive adverse conditions (Hezayen *et al*., 2001). Eukaryotic organisms such as *Cnidaria* are also known to produce γ-PGA. These marine animals have nematocysts which are stinging cells which they use explosively for prey capture, locomotion, and protection. Large amounts of γ-PGA helps to trigger this explosive reaction (Weber, 1990). As earlier mentioned, γ-PGA has also been associated with the neurons of mice (Edde *et al*., 1990) where it is considered to play a role in the regulation of microtubule dynamics by modifying the interaction of tubulin with tubulin-associated proteins and Ca^{2+} (Edde *et al*., 1990). *Bacillus amyloliquefaciens* C06 uses γ-PGA to improve both its ability to form biofilm and motility by causing cells to stick together in a coordinated pattern and absorbing essential nutrients needed for motility from the environment respectively (Liu *et al*., 2010) while *B. licheniformis* WX-02 produced γ-PGA to increase its survival when exposed to high osmotic stress (Wei *et al*., 2010).

2.3 Mechanism of synthesis of poly-γ-glutamic acid

Poly-γ-glutamic acid (γ-PGA) synthesis is a ribosome independent mechanism that requires enzyme enhancement. Two specific types of ribosome independent amino acid polymerization have been suggested up to this present time (Ashiuchi, 2010). One is the thiotemplate mechanism (Kleinkauf and Dohren, 1996) and the second mechanism involves the action of amide ligation (Ashiuchi and Misono, 2002).

The catalysis of the thiotemplate mechanism is carried out by non-ribosomal peptide synthetases (NRPSs) that are usually referred to as multienzyme systems because of the coexistence of several domains (Ashiuchi, 2010). These domains include a typical adenylation domains, peptidyl carrier domains, specific epimerization domains, *N*-methylation domains and thiolase domains (Vater *et al*., 1985, Stein *et al*., 1995 and Kleinkauf and Dohren, 1996). These domains can be distinguished by: the presence of NRPSs consensus sequences, production of similarly small polyamides, occasional controversy of stereochemistry between a polymer produced and the substrate based on the strict choice of L-amino acid substrates (Ashiuchi, 2010) and the generation of AMP and PPi by concurring ATP hydrolysis.

The catalysis of the amide ligation mechanism is carried out by typical amide ligases which include murein-biosynthetic enzymes (Evenland *et al*., 1997) or by ATP-grasp peptide synthetases which includes γ-glutamylcysteine synthetase and D-alanyl-D-ananyl ligase (Galperin and Koonin, 1997). These ligases can also be distinguished by various instances including generation of ADP and Pi as a result of ATP hydrolysis, presence of consensus sequences typical for amide ligases or similar to ATP-grasp peptide synthetases, and a compliance of stereochemistry between a polymer synthesized and the substrates as a result of lack of isomerization action on the amino acid residues in a growing chain (Ashiuchi, 2010).

The cost of producing γ-PGA is one of the major challenges of making its usage applicable in the industry. According to Sung *et al.* (2005b), γ-PGA is several tens to hundred fold more expensive than the conventional materials it is envisioned to replace. Reducing the cost of production is the only foreseeable solution to this problem (Bhat, 2012). Designing mass production systems for γ-PGA would be a major step towards achieving effective results. To accomplish this, detailed knowledge of how different factors affect the yield of production as

well as information about genes and enzymes involved in γ-PGA production would be helpful in manipulating organisms for more efficient production of γ-PGA.

In recent years, there has been a rise in research in this direction (Ashiuchi & Misono, 2002, Buescher & Margaritis, 2007, Candela & Fouet, 2006 and Sung *et al.*, 2005b) and genes that play a role in every step of γ-PGA production have been identified. This section attempts to provide an understanding of the current knowledge of the mechanism of γ-PGA synthesis.

A biosynthetic pathway for the production of γ-PGA has been proposed (**Fig. 2**). L-glutamic acid units that make up γ-PGA can be derived from two sources. It can either be obtained via the glutamic acid biosynthetic pathway exogenously or endogenously. Endogenous production of L-glutamic acid requires conversion of a carbon source via acetyl-CoA and TCA cycle intermediates. α-ketoglutaric acid from the TCA cycle serves as a direct precursor of glutamic acid synthesis (Ko & Gross, 1997 and Shih & Wu, 2009). Exogenous L-glutamic acid can be converted to L-glutamine with the help of the enzyme glutamine synthetase. L-glutamine is a precursor of γ-PGA as well. The process of γ-PGA synthesis can be seen to have 4 distinct stages – γ-PGA racemisation, γ-PGA polymerization, γ-PGA regulation and γ-PGA degradation

2.3.1 γ-*PGA racemisation*

As mentioned earlier, γ-PGA can have L or D or both L and D enantiomers of glutamic acid in varying amount. To incorporate D-glutamic acid into the growing chain of γ-PGA, it needs to be obtained from L-glutamic acid (exogenously supplied or produced *de novo*) by a racemisation reaction.

B. subtilis has two homologues of glutamate racemase, *racE/glr* and *yrpC* (Ashiuchi *et al*., 1998 & Ashiuchi *et al*., 1999b). The functions of these genes are still uncertain as there are differing reports on the importance of each gene (Ashiuchi *et al*., 2003b, Kada *et al*., 2004 &

Kimura *et al*., 2004). *RacE* is a cytosolic enzyme with a high selectivity for glutamic acid and a preference for L-glutamic acid (Buescher & Margaritis, 2007 & Thorne *et al*., 1955). According to Kimura *et al*. (2004), *racE* was only important for growth in complex medium whereas *yrpC* was found to be active when the cells were grown on minimal medium. Neither of the two glutamate racemase genes was found responsible for the synthesis of γ-PGA even though both seemed essential D-glutamate catabolism (Kimura *et al*., 2004).

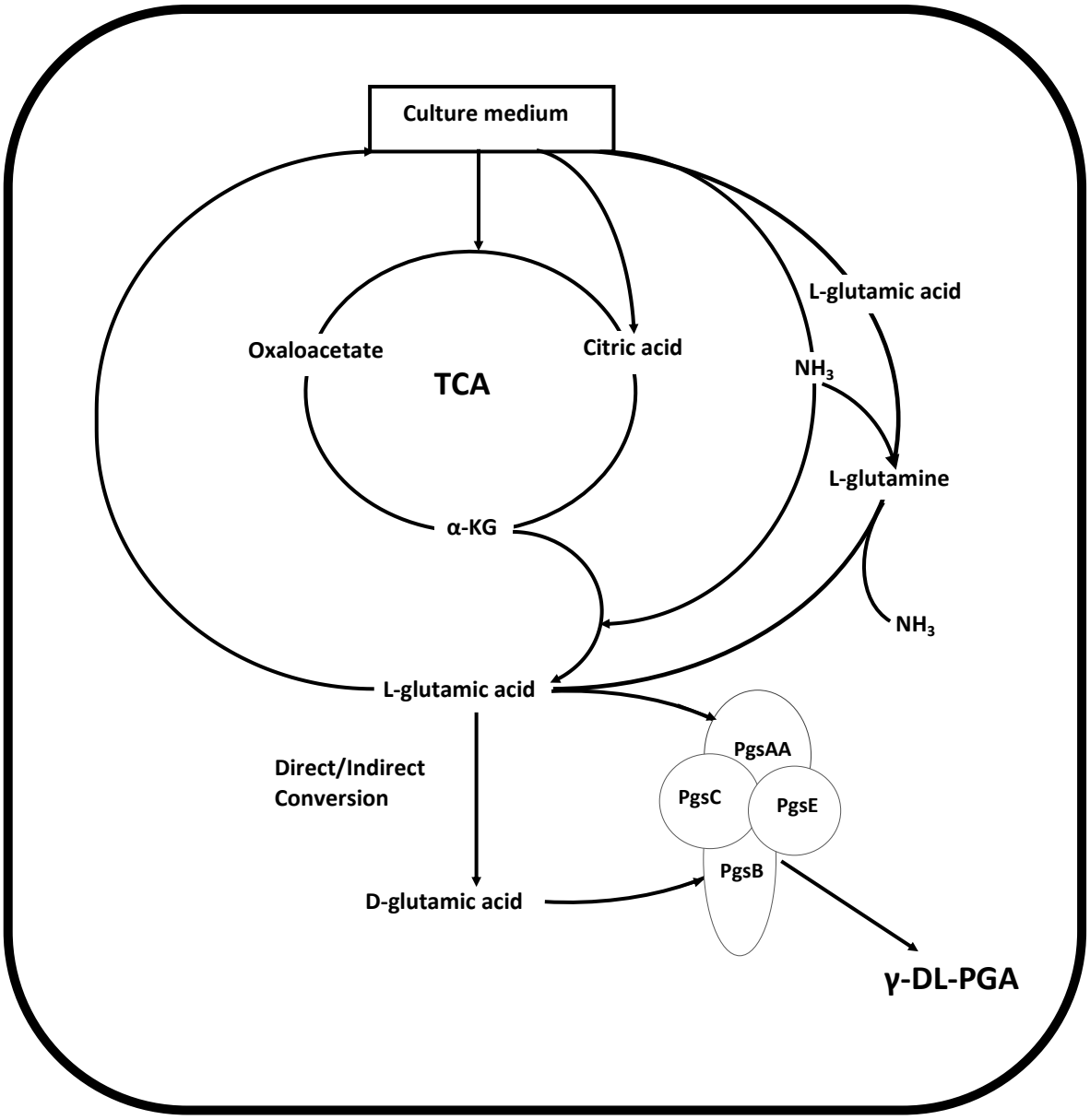


Figure 2: A proposed pathway for the synthesis of γ-PGA in *Bacilli*. TCA = Tricarboxylic acid cycle Adapted from Ho *et al*., 2006, Buescher & Margaritis, 2007 & Shih & Wu, 2009

On the contrary, Kada *et al*. (2004) found *glr* to be essential for converting L-glutamate into D-glutamate for the synthesis of γ-PGA and peptidoglycan as well as growth in *B. subtilis*. Research has however shown that Mn^{2+} affects the enantiomeric composition of γ-PGA (Cromwick & Gross, 1995a, Cromwick & Gross, 1995b, Perez-Camero *et al*., 1999 & Wu *et al*., 2006). Ashiuchi *et al*. (2004) showed that Mn^{2+} affected the enantiomeric composition by altering the expression of the *glr* gene. The ratio between L- and D-glutamate in *B. licheniformis* γ-PGA also depends on the concentration of Co^{2+} and Zn^{2+}. Other researchers have also confirmed the dependence of enantiomeric composition of γ-PGA on Mn^{2+} in both *B. licheniformis* and *B. subtilis* (Cromwick & Gross, 1995a & Wu *et al*., 2006).

2.3.2 γ-*PGA polymerization*

In the case of *B. anthracis*, the genes involved in γ-PGA synthesis lie on a large plasmid as opposed to other *Bacillus* species where the genes are present on the chromosome (Shih & Van, 2001 & Ashiuchi *et al*., 2001a).

When γ-PGA is surface associated (as in the case of *B. anthracis*), the "*cap*" (capsule) genes are required for its production. On the other hand, for γ-PGA that is released, the "*pgs*" (polyglutamate synthase) genes come into action (Candela & Fouet, 2006). Both the *cap* and *pgs* gene sets have at least 4 genes – the *cap* or *pgs B*, *C*, *A* and *E*. (**Fig. 3**). The *pgsBCA* genes of *B. subtilis* IFO3336 (*B. natto*) are homologous to *capBCA* genes of *B. anthracis* (Shih & Van, 2001 & Makino *et al*., 1989). *pgsBCA* has been identified as the sole machinery responsible for γ-PGA synthesis in *Bacillus* species. To prove this, Sung *et al.* (2005b) disrupted *pgsBCA* genes in *B. subtilis* (chungkookjang) creating *pgsBCA* null mutants incapable of γ-PGA production. In contrast, Urushibata *et al.* (2002a) are of the opinion that only *pgsB* and *pgsC* are required for the production of γ-PGA. The role of *pgsE* in the production of γ-PGA is dispensable since *pgsB*, *pgsC* and *pgsAA* at high concentration was able to form a complex capable of producing γ-PGA even in the absence of *pgsE* (Candela *et*

al., 2005). On the contrary, Yamashiro *et al*. (2011) found *pgsE* to be essential in γ-PGA production as its introduction into the production medium in the presence of Zn^{2+} tripled the production rate of γ-PGA from *B. subtilis* subsp. *Chungkookjang*. Candela *et al.* (2005) showed that CapE (a 47 amino acid peptide) is also responsible and essential for the production of γ-PGA as it appeared to interact with CapA. The unique membrane bound pgsBCA complex is highly unstable and hydrophobic, which makes isolation of this complex challenging.

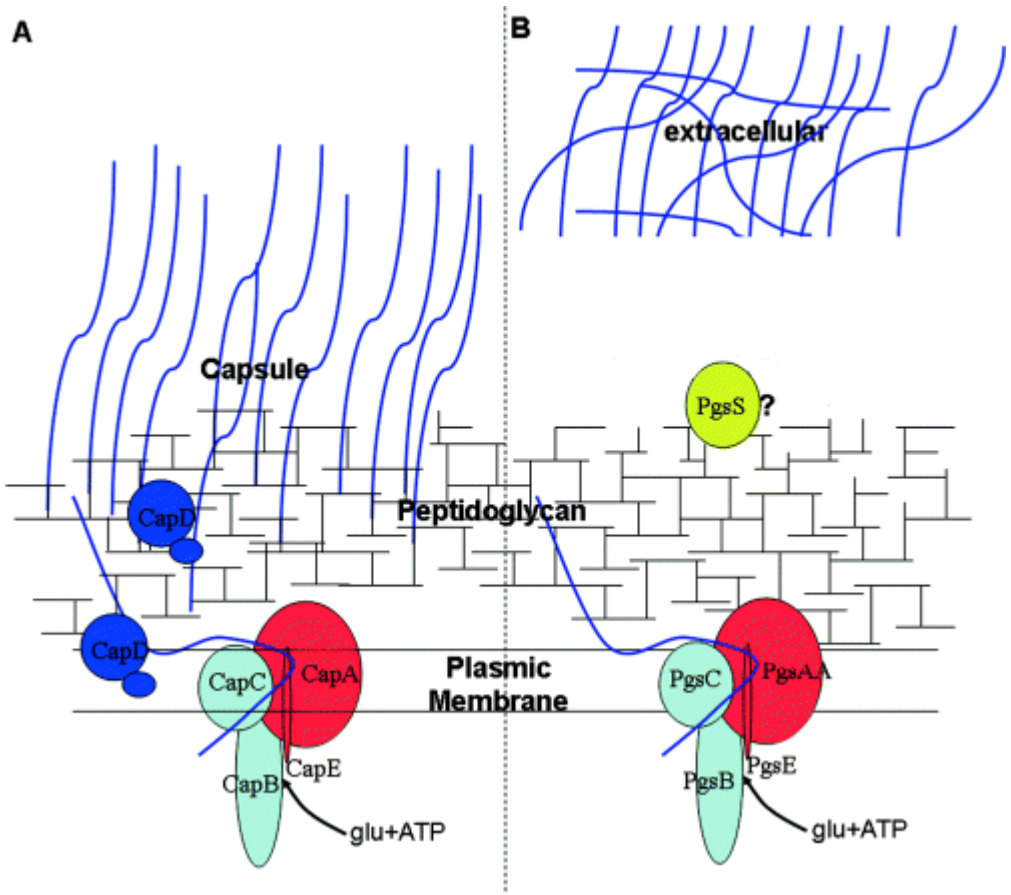


Figure 3: Schematic representation of γ-PGA synthesis complex. The genetic setup required for γ-PGA synthesis when it is detached (*B. subtilis* and *B. licheniformis*) and when it is attached (*B. anthracis*) Source: Candela & Fouet, 2006

As mentioned earlier, the mechanism of polymerisation has been shown to be dependent on ATP. The phosphoryl group of ATP is first transferred to a terminal carboxyl group of elongated γ-PGA through substrate dependent ATP hydrolysis (Sung *et al.*, 2005b). Then, due to a nucleophilic attack of an amino group of glutamic acid on the phosphorylated

carboxyl group, an amide linkage is formed. This reaction continues to polymerize γ-PGA at the active site of the synthetase complex (pgsBCA). PgsB and PgsC together form most parts of the complex's catalytic site whereas PgsA seems to remove the elongated chain from the active site so that the next monomer could be added and may also be involved in transporting γ-PGA (Ashiuchi *et al*., 2001b & Urushibata *et al*., 2002a). Activity of PgsBCA was found to be dependent on Mg^{2+} (Ashiuchi *et al*., 2004). Transportation of γ-PGA outside the cell could be facilitated by a less compact cell membrane with shorter phospholipids (Buescher & Margaritis, 2007).

2.3.3 γ-*PGA regulation*

γ-PGA synthesis in *B. subtilis* (*natto*) is said to be regulated by the ComP–ComA signal transduction system (Tran *et al*., 2000). Stanley & Lazazzera (2005) further identified a two part system DegS-DegU, DegQ and SwrA as unusual regulators of γ-PGA production in addition to ComPA. The regulatory effects of DegSU, DegQ and ComPA seem to be transcriptional in response to quorum sensing, osmolarity and phase variation signals while that of SwrA seems to be post-transcriptional (Stanley & Lazazzera, 2005). Osera *et al*. (2009) examined the relationship between SwrA and DegU and discovered that presence of both SwrA and phosphorylated DegU (DegU∼P) is required to fully activate *pgs* operon and in turn γ-PGA production since the effect of either gene on both *pgs* transcription and γ-PGA production is negligible. In contrast, Ohsawa *et al*. (2009) showed that high degree of DegU∼P can directly activate *pgs* expression in place of *swrA* and high levels of *degQ* but still found *swrA* necessary for γ-PGA production under certain experimental conditions.

To identify the role of DegQ in γ-PGA synthesis, *degQ* gene was knocked out in *Bacillus subtilis* (natto) and suppressor mutants capable of producing γ-PGA synthesis in the absence of *degQ* were isolated and used (Do *et al*., 2011). This was compared with domestic strain *B. subtilis* 168 that is unable to produce γ-PGA due to low transcription of the biosynthetic *pgs*

operon even though it has a higher genetic competency than wild strain *B. subtilis* (natto) (Osera *et al*., 2009 & Stanley & Lazazzera, 2005). Do *et al*. (2011) found that an alteration in *degQ* drastically prevented the synthesis of γ-PGA in *B. subtilis* (natto) and downregulated the production of degradation enzymes which is in line with the report of Stanley & Lazazzera (2005). The *degQ* gene of *Bacillus subtilis* (natto) is therefore important for the synthesis of γ-PGA (Do *et al*., 2011).

2.3.4 γ-*PGA degradation*

There are enzymes that have been shown to be associated with the degradation of γ-PGA. γ-glutamyl-transpeptidases (GGT) are enzymes that breakdown the transfer of a γ-glutamyl group from a donor species to acceptor (peptides and amino acids) species by developing an intermediate γ-glutamyl enzyme in a transpeptidation reaction (Morelli *et al*., 2014). The enzyme has the ability to perform exo-hydrolase activity towards γ-PGA and the released glutamic acid is used by the bacterium as a source of carbon and nitrogen (Kimura *et al*., 2004 & Morelli *et al*., 2014). *pgdS* (also *pgsS*) is located downstream and in the same orientation as the *pgsBCA* operon (Ashiuchi *et al*., 2003a, Buescher & Margaritis, 2007, & Suzuki & Tahara, 2003). It is known to encode the enzyme, γ-glutamyl-hydrolase (PgsS) which is responsible for γ-PGA degradation and cleaves γ-PGA between two D-glutamates (Ashiuchi *et al*., 2003b & Candela & Fouet, 2006).

CapD, a γ-glutamyl-transpeptidase is a part of the GGT family and is required for the covalent anchoring of γ-PGA to the peptidoglycan as well as acting as a depolymerase (Candela & Fouet, 2005). CapD cleaves the γ-PGA and transfers it to either an acceptor molecule or H_2O resulting in transpeptidation or hydrolysis respectively (Candela *et al*., 2014). Since γ-PGA can be either anchored to the bacterial surface or released, whilst CapD catalyses the anchorage of γ-PGA to peptidoglycan, PgsS catalyses the release of γ-PGA (Candela & Fouet, 2006). The strains that have *pgsBCA* but do not produce γ-PGA, do so

because the genes are not translated and not because an inactive gene product is produced (Urushibata *et al.*, 2002b).

Recently Yao *et al*. (2009) investigated the presence and activity of γ-PGA depolymerase enzyme in *B. subtilis* NX-2 which is responsible for the depolymerisation of γ-PGA in batch culture. The enzyme was seen to be active extracellularly in the culture and was shown to be an endo-hydrolase. The gene encoding the enzyme was *ywtD* (*pgsS*). The YwtD protein was obtained in purified form after the gene was cloned and expressed in *E. coli*. The enzyme was active between a temperature range of 30-40 °C and a pH range of 5-8. At the optimal pH and temperature (5 and 30 °C respectively), γ-PGA molecular weight (M_w) reduction from 1000 to 20 kDa was observed and dispersity decreased as a function of depolymerisation time. The enzyme was also seen to be active extracellularly during the late stationary phase. The study demonstrated a mild method for controlled reduction of molecular weight and could be used as a better alternative to physical and chemical methods of degradation.

2.4 Conformation and enantiomeric composition of γ-PGA

γ-PGA can exist in different conformational states namely 'alpha helix, beta sheet, helix to random coil transition, random coil and enveloped aggregate' based on the environmental conditions (Ho *et al*., 2006). The conformational state of γ-PGA can change depending on a number of factors such as pH, polymer concentration and ionic strength. It has been shown that γ-PGA purified from *B. licheniformis* can exist in different conformational states depending on the concentration of γ-PGA and the pH of the solution (He *et al*., 2000). γ-PGA takes up a conformation based largely on α-helices at low concentration (0.1% w/v) and when the pH is below 7.0, while a β-sheet-based conformation predominates at higher pH. The β-sheet conformation seems to expose the negative charges of γ-PGA very efficiently (Candela and Fouet, 2006). γ-PGA conformation is sensitive to small changes in specific factors, for

instance, changes in PGA side-chain ionisation can have a pronounced effect on the conformation (Shih & Van, 2001 and Tiffany & Krimm, 1969).

γ-PGA also varies in enantiomeric composition (**Table 2.1)**. The enantiomeric composition of γ-PGA determines how γ-PGA is extracted after fermentation. If γ-PGA contains only L or D enantiomers then it dissolves in ethanol. However, if L and D forms are in equimolar amounts, then γ-PGA precipitates in ethanol (Candela & Fouet, 2006).

Table 2.1: Enantiomeric composition of PGA producing organisms

γ-PGA producers	**PGA Composition**		**References**
	$_{D}$-Glutamates (%)	**$_{L}$-Glutamates (%)**	
Bacillus anthracis	100	-	(Hanby & Rydon, 1946)
Bacillus subtilis ATCC 9945a	~10 - ~100*	~10 - ~90*	(Thorne & Leonard, 1958)
(now *Bacillus licheniformis* 9945a)			
Bacillus megaterium	30	70	(Sung *et al*., 2005b)
Bacillus subtilis	70	30	(Ashiuchi *et al.*, 2003a)
Bacillus chungkookjang	70	30	(Sung *et al*., 2005b)
Staphylococcus epidermidis	40	60	(Kocianova *et al.*, 2005)
Natrialba aegyptiaca	-	100	(Sung *et al*., 2005b)
Bacillus subtilis BS 62	60	40	(Choi *et al.*, 2004)
Bacillus licheniformis A35	80-83*	17-20*	(Cheng *et al*., 1989)

* Dependent on the concentration of Mn^{2+}

Furthermore, various γ-PGA obtained from *Bacillus* species normally have different molecular masses ranging from 10 to 1000kDa. A high molecular mass PGA of about 1000kDa is produced by *Bacillus megaterium* (Ashiuchi *et al*., 2003a) while *Bacillus halodurans* synthesizes a low molecular weight (approximately 10kDa) γ-(L)-PGA (Aono, 1987).

It is necessary to determine the effect of the enantiomeric composition of γ-PGA on its behaviour and efficacy for a specific application (Bhat 2012). When γ-PGA with different enantiomeric compositions was tested for its antifreeze properties, it was seen that the antifreeze activity of γ-PGA was not affected by its enantiomeric composition, but only by its

molecular weight (Shih *et al*., 2003). The antifreeze property was seen to be affected by the concentration of cations in the order of $Mg^{2+}>>Ca^{2+}$ $Na^{+}>>K^{+}$ (Bhat, 2012).

Culture conditions seem to have effect on the enantiomeric composition and molecular weight of γ-PGA, both of which can alter its properties (Shih and Wu, 2009). It would be interesting to see how these properties affect specific applications. It is therefore important to know the conformational state of γ-PGA when it is employed in various applications since a small change in environmental conditions can alter the properties of γ-PGA to a large extent (Bhat, 2012).

2.5 Production of γ-PGA by microbial fermentation

Increasing numbers of researchers have focused on the production of γ-PGA from bacterial fermentation. PGA has a molecular weight of less than 10,000 Da when it is synthetically produced (α-PGA) which limits its application. However, when PGA is produced by microbial fermentation (γ-PGA), it has a molecular weight greater than 10,000 Da and usually ranges from about 100 kDa to greater than 1,000 kDa (Bajaj *et al*., 2009, Park *et al*., 2005 and Richard & Margaritis, 2003).

Although the microbial production of γ-PGA has since been established, the cost of production which ultimately affects the market price {≈ £137 per 100mg high purity Na salt γ-PGA (Sigma Aldrich)} of the polymer is presently very high and this is a major limitation to the widespread application of the polymer. Based on this, most research on microbial production of γ-PGA has focused on the optimisation of growth conditions with the potential to produce high yield, specific enantiomeric composition and desired molecular weight of γ-PGA at reduced cost. The medium used to produce γ-PGA by various bacteria is important because it directly affects the properties of γ-PGA. For instance, according to Sung *et al*. (2005b), in *B. subtilis (chungkookjang*) altering the NaCl concentration in the medium

between a particular range of 0 and 100g/L could regulate the molecular weight of γ-PGA. Likewise, the molecular weight of γ-PGA also differs based on the strains used. For example *Bacillus sp.* RKY3 and *B. subtilis* (chungkookjang) produce γ-PGA of 10-50 kDa and in excess of 10,000 kDa respectively even under similar cultural conditions (Buescher and Margaritis, 2007).

High molecular weight γ-PGA is very promising for various industrial applications. However, it is generally diverse in its molecular structure and during the fermentation it is co-produced with different polysaccharides and other biopolymers. Production of γ-PGA from *Bacillus subtilis* (natto) with a molecular weight higher than 2 x 10^6 Da has proved to be challenging for various reasons: γ-PGA synthesis or elongation is sometimes coupled with degradation of γ-PGA towards the later stages of fermentation; the PGA synthetase complex itself is not stable. However, *B. subtilis* subsp. *chungkookjang* cultivated in a medium with high concentration of ammonium sulphate has been found to produce super-high-molecular-weight γ-PGA without the aforementioned problems (Park *et al*., 2005). The high molecular weight γ-PGA averaging 2 x 10^6 Da was obtained without the presence of any by-products and was richer in L-glutamate than its D-enantiomer. Park and co-workers also managed to isolate γ-PGA which had a molecular weight higher than 2 x 10^6 Da but it was difficult to accurately measure such a high molecular weight γ- PGA. It was thought to be around 7 x 10^6 Da.

According to Shih and Van, (2001), γ-PGA producing bacteria have been divided into two groups depending upon their nutrient requirement for γ-PGA production – those that require L-glutamic acid in the medium and those that do not require L glutamic acid. The L-glutamic acid dependent bacteria include *B. subtilis* chungkookjang (Ashiuchi *et al*., 2001a), *B. licheniformis* 9945a (Birrer *et al*., 1994) *B. subtilis* CGMCC 0833 (Wu *et al*., 2010b), *B. licheniformis* NK-03 (Cao *et al*., 2010) and B. *subtilis* (natto) ATCC 15245 (Bhat *et al*.,

2013) while the non L-glutamic acid dependent producers include *B. subtilis* C1 (Shih *et al*., 2005), *B. amyloliquefaciens* LL3 (Cao *et al*., 2011) and *B. subtilis* C10 (Zhang *et al*., 2012). For L-glutamic acid dependent bacteria, the PGA yield increases with an increase in the L-glutamic acid concentration in the medium. However, these bacteria can produce γ-PGA even in the absence of exogenously supplied L-glutamic acid due to the resource of L-glutamic acid obtained through the *de novo* pathway (Buescher & Margaritis, 2007 and Kunioka & Goto, 1994). The L-glutamate independent producers are more desirable for industrial γ-PGA production than the glutamate dependent producers due to their low cost of production and simple fermentation process (Cao *et al*., 2011). However, their industrial utilization is limited due to lower γ-PGA productivity compared to those of L-glutamate dependent producers. This has led to the development of genetically engineering non-glutamate dependent producers such as *B. amyloliquefaciens* NK-1 (Feng *et al*., 2014) as well as laboratory strains like *B. subtilis* MA41 (Ashiuchi *et al*., 2006), *E. coli* (Cao *et al*., 2013) and *B. subtilis* 168 (Scoffone *et al*., 2013) for high γ-PGA productivity.

2.6 Production with *Bacillus* species

2.6.1 *Bacillus licheniformis*

B. licheniformis has been widely used for γ-PGA production. *Bacillus licheniformis* 9945a (NCIM 2324) is a well-known L-glutamate dependent strain of *B. licheniformis* which has been used for γ-PGA production. As mentioned earlier, efforts have been made to optimize production of γ-PGA with a vision to obtain maximum yield. Bajaj *et al.* (2008) optimized the production of γ-PGA using *B. licheniformis* NCIM 2324 in solid state fermentation. Using the "one factor at a time" method, they investigated the effect of solid substrates, moisture content, pH, C and N sources, amino acids and TCA cycle intermediates on the production of γ-PGA. Their optimized media gave a maximum yield of 98.64 g/kgds of γ-PGA in solid fermentation.

Bajaj and colleagues also optimized the production of γ-PGA with *B. licheniformis* NCIM 2324 using the "one factor at a time" method (Bajaj *et al*., 2009). The optimum nutrient concentrations were devised with the help of response surface methodology. These were then tested experimentally. The yield obtained with the devised medium (glycerol, 62.4; citric acid, 15.2; ammonium sulphate, 8.0; L-glutamic acid, 20) g/l was 26.12 g/l as opposed to the basal medium (5.27 g/l). The molecular weight of γ-PGA thus obtained was 2.1 x 10^5 Da.

Based on the above work, Bajaj and Singhal developed a process that achieved a productivity of 35.75 g/l of γ-PGA using *B. licheniformis* NCIM 2324 (Bajaj & Singhal, 2009). This was achieved by feeding *B. licheniformis* NCIM 2324 with some metabolic precursors for γ-PGA production, (0.07 g/l L-glutamine and 1.46 g/l α-ketoglutaric acid) in addition to the basal medium. The yield of γ-PGA obtained was 35.75 g/l in contrast to 26.12 g/l when no metabolic precursors were added. The added precursors helped in the better utilisation of L-glutamic acid.

Bacillus licheniformis A13, an exogenous glutamate independent producer presented a γ-PGA yield of 28.2 g/l in an optimised medium (g/l – glucose, 50; NH_4Cl, 3; yeast extract, 2; $MgSO_4.7H_2O$, 0.8; NaCl, 0.8; $CaCl_2.2H_2O$, 0.00084; K_2HPO_4, 6.4; $FeSO_4.4H_2O$, 0.006; 0.1 ml of trace element solution and 25 ml culture volume) devised using the Plackett–Burman design after 72 hours of inoculation (Mabrouk *et al*., 2012). Results showed that yeast extract and medium volume were the two factors affecting the γ-PGA production.

B. licheniformis WX-02, a halotolerant γ-PGA producer was investigated for the effect of NaCl concentration on the production of γ-PGA in a modified E medium where glycerol was replaced with glucose (g/l - L-glutamic acid 20, glucose 20, citric acid 12, NH_4Cl 7, K_2HPO_4 0.5, $MgSO_4.7H_2O$ 0.5, $FeCl_3.6H_2O$ 0.04, $CaCl_2.2H_2O$ 0.15, $MnSO_4.H_2O$ 0.104, pH 6.5) (Wei *et al*., 2010). Results showed that the production of γ-PGA in *B. licheniformis* WX-02 is salt inducible as γ-PGA yield increased from 2.16±0.09 g/l with 0% NaCl to 13.86 g/l with 8%

NaCl concentration. The molecular size of γ-PGA however, decreased with increase in NaCl concentration.

2.6.2 *Bacillus subtilis*

Bovarnick first showed that γ-PGA is freely secreted into the medium after fermentation of *Bacillus subtilis* in 1942 (Bovarnick, 1942). Following this, considerable research (Huang *et al*., 2011, Scoffone *et al*., 2013 and Shih *et al*., 2005) have been carried out into the production of γ-PGA using *B. subtilis.*

γ-PGA production was recently evaluated by Scoffone and colleagues (Scoffone *et al*., 2013). This was done by inactivating *pgdS* and *ggt* (genes for two important γ-PGA degrading enzymes) in the laboratory strain – *B. subtilis* 168. The effects of single mutations (either *pgdS* deletion or *ggt* deletion) and double mutation (both *pgdS* and *ggt* deletion) on γ-PGA production were assessed. Results revealed that single mutations had no significant improvement on γ-PGA yield whereas a double fold increase (>40 g/l) was observed in the double mutant strain compared to wild type strain. However, number average molecular weight (M_n) and weight average molecular weight (M_w) of double mutant produced γ-PGA was lower compared to those of single mutant strains and wild strain. *pgdS* mutant strain presented the highest molecular weight (>3 MDa) probably due to reduced endo-degradation activities.

High yield, cost-effective and large scale production of γ-PGA from *B. subtilis* ZJU-7 (*B. subtilis* CGMCC1250) has been reported by Huang *et al*. (2011). Their findings showed that 40 g/l of yeast extract, 30 g/l of L-glutamate and 20 g/l initial glucose as well as keeping glucose concentration in the range of 3–10 g/l through a fed-batch approach greatly improved the production of γ-PGA 1.4 to 3.2 fold compared to those in batch fermentation. An overall γ-PGA concentration of 101.1 g/l and a productivity of 2.19 g/l were recorded.

B. subtilis CCTCC202048 was investigated for production of γ-PGA in solid state fermentations (SSF) by Jian *et al* (2005). Maximum γ-PGA production (83.61 g $kgds^{-1}$) was obtained when both soybean cake powder and wheat bran were used as mixed substrates in a ratio of 11:9 w/w in addition to glutamate (40.14 g/kg), citric acid (18.50 g/kg), NH_4NO_3 (20.05 g/kg) and mineral salts ($MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $FeCl_3 \cdot 6H_2O$ and $MnSO_4 \cdot H_2O$). SSF can be useful for the production of large scale γ-PGA due to its high productivity and low production costs.

Shih and co-workers devised a novel glycerol-γ-PGA derivative using *B. subtilis* C1 without L-glutamate in the medium (Shih *et al*., 2005). *B. subtilis* C1 was dependent on both citric acid and glycerol. If either of these were absent in the medium, production of γ-PGA was negligible. Results showed that the molecular weight of the conjugate (1 x 10^7 Da) was higher than the super-high-molecular weight γ-PGA produced by Park *et al.* (2005) due to the presence of glycerol. The ratio of γ-PGA to glycerol in the conjugate was 10:1. The conjugate had a higher concentration of D-glutamic acid units (97%) than L-glutamic acid units. Interestingly, Mn^{2+} did not seem to affect the enantiomeric composition of the glycerol-γ-PGA conjugate.

In contrast Mn^{2+} affected the stereochemical and enantiomeric composition of γ-PGA produced from *B. subtilis* NX-2 (Wu *et al.*, 2006). When the concentration of Mn^{2+} increased from 0 to 0.09 g/L, the proportion of D-glutamate increased from 18 to 77%. Mn^{2+} seemed to affect the stereo chemical properties of γ-PGA by altering the activity of glutamate racemase.

The effects of five organic acids (succinic acid, citric acid, fumaric acid, oxalic acid and acetic acid) on γ-PGA production by *Bacillus subtilis* C10, a glutamic acid-independent γ-PGA producer was investigated (Zhang *et al*., 2012). Results revealed that citric acid or oxalic acid could greatly increase the productivity of γ-PGA by regulating the bioactivities of some key enzymes. The highest volumetric yield of γ-PGA (27.7 g/L) was achieved by

adding 20 g/L citric acid into the fermentation medium (80 g/L glucose, 10 g/L NH_4Cl, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L K_2HPO_4, 0.04 g/L $FeCl_3 \cdot 6H_2O$, 0.104 g/L $MgSO_4 \cdot H_2O$ and 0.11 g/L $CaCl_2$, pH 7.2) when cultivated at 32 °C and 200 rpm for 24–32 h.

2.6.3 *Production using other bacteria*

γ-PGA synthetase genes *pgsBCA* and *racE* from a L-glutamate-dependent γ-PGA producer *B. licheniformis* NK-03 and a non L-glutamate-dependent γ-PGA producer *B. amyloliquefaciens* LL3 were cloned and co-expressed in *E. coli* JM109 for evaluation of γ-PGA productivity (Cao *et al*., 2013). Their findings showed that the *pgsB and pgsC* of both strains are highly similar with 93.13 and 93.96% resemblance while the *pgsA* and *racE* presented 78.53 and 84.5% similarity respectively. Results further showed that the four engineered strains primarily produced γ-PGA in both glucose and L-glutamate medium after culturing for 24 hours. The *PgsBCA* developed from *B. amyloliquefaciens* LL3 seemed to possess better catalytic activity than that of *B. licheniformis* NK-03 irrespective of the harbouring vector as the quantity of γ-PGA produced by *B. amyloliquefaciens* LL3-*pgsBCA* was higher than that *B. licheniformis* NK-03-*pgsBCA*. There was significant improvement in productivity and D-isomer content of γ-PGA from *B. amyloliquefaciens* LL3-derived *pgsBCA* & *racE* and *B. licheniformis* NK-03-derived *pgsBCA* & *racE* than in γ-PGA from *B. amyloliquefaciens* LL3-derived *pgsBCA* and *B. licheniformis* NK-03-derived *pgsBCA* showing that the incorporation of *racE* enhanced the productivity of γ-PGA as well as its D-isomer.

In a study by Xu *et al.* (2005b), *B. subtilis* was co-cultured with *Corynebacterium glutamicum* using glucose and sucrose as mixed carbon source in order to avoid the addition of exogenous L-glutamic acid and this turned out to be helpful in reducing fermentation time and production cost. The average molecular weight of γ-PGA thus produced was 1.24×10^6 Da.

B. anthracis is known to produce a pure D-enantiomer of γ-PGA (Zwartouw & Smith, 1956). The major difference in the mechanism of production of *B. anthracis* compared to other *Bacillus* species is that it does not secrete γ-PGA into the medium, but instead, it is peptidoglycan bound. This makes the recovery and purification procedure of γ-PGA difficult. More importantly, industrial production of γ-PGA from *B. anthracis* is not viable owing to its toxicity. In fact, the anchored γ-PGA is responsible for the non-immunogenic capsule of *B. anthracis* which has been associated with the lethal toxin (LT) (Ezzell *et al*., 2009). Hence to render *B. anthracis* immunogenic, its *cap* gene responsible for the anchoring of γ-PGA onto its surface, needs to be targeted (Candela & Fouet, 2006).

B. thuringiensis serovar Monterrey strain BGSC 4AJ1 was reported to produce a γ-PGA capsule similar to that of *B. anthracis* (Cachat *et al*., 2008). *B. thuringiensis* serovar Monterrey strain BGSC 4AJ1 and *B. anthracis* (Ames) had four common alleles - gmk-1, pta-1, pur-1 and tpi-1 while the other three, glpF-57, ilvD-52 and pycA-52 contrasted in 2, 2 and 3 nucleotides respectively. The γ-D-PGA producing genes are similar to those of *B. anthracis* and are present on a plasmid (pAJ1-1). The discovery of a γ-PGA capsule in this strain of *B. thuringiensis* is an indication that the bacteria could be pathogenic under certain conditions.

The essential *pgsBCA* synthase complex was inserted into tobacco leaves via *Agrobacterium* infection (Tarui *et al*., 2005). γ-PGA was produced in plant tissue where all three *pgsB*, *pgsC* and *pgsA* genes were expressed and about 600μg of γ-PGA was produced per gram of leaf material.

2.7 Extraction and recovery of poly-γ-glutamic acid (γ-PGA)

The recovery of γ-PGA from the culture broth is quite elementary because γ-PGA is produced extracellularly by *Bacillus* spp. Three basic types of methods namely: precipitation by reduction of water activity using organic solvents, precipitation by formation of complex with cations (Cu^{2+}, Al^{3+}, Cr^{3+}, and Fe^{3+}) and filtration can be used in the recovery of γ-PGA (Buescher and Margaritis, 2007 and Shih and Van, 2001). The first step usually involved in all types of recovery is the extraction of biomass by centrifugation or filtration in a 0.45 μm filter (Yoon *et al*., 2000, Goto and Kunioka, 1992 and Shih *et al*., 2002). Secondly, the filtrate can be precipitated either by using organic solvents such as ethanol, methanol or 1-propanol by reducing the moisture content in the cell-free broth (Kubota *et al*., 1993) or by metal-induced precipitation. Even though precipitation using organic solvents, particularly ethanol is the most widely used method for recovering γ-PGA, there are a number of difficulties associated with this method. These include co-precipitation of unwanted extracellular polysaccharides present in the broth, large volumes of organic solvents which may be expensive and lead to environmental concerns and inability of the organic solvent to completely precipitate γ-PGA from the broth (Manocha and Margaritis, 2010). Another method requires separation of γ-PGA by a series of filtration which involves membrane filtration and buffer changing procedures (Buescher and Margaritis, 2007). The number of filtration steps required depends on the expected level of purity. This method is also limited by its speed as it is a slow process and the filtration membranes need frequent replacement as a result of quick membrane fouling. These disadvantages have led to the development of "copper-sulphate induced precipitation of γ-PGA", a type of metal-induced precipitation by Manocha and Margaritis (2010). This technique presents an effective and cost-effective recovery process where γ-PGA can be selectively precipitated from the fermentation broth in the absence of expensive organic solvents and membrane filtration.

2.8 Identification and characterization of poly-γ-glutamic acid

Molecular characterization of γ-PGA is essential in order to evaluate its properties and hence its potential application areas.

2.8.1 *Identification using Fourier transform infra-red (FT-IR) spectroscopy*

Fourier Transform Infra-Red Spectroscopy (FT-IR) technique which uses an infrared absorption spectrum is used for product identification. FT-IR spectroscopy produces infrared spectra of γ-PGA with peaks corresponding to a specific bond in the compound. According to Ho *et al*. (2006), the infrared spectra of γ-PGA (free acid form) and γ-polyglutamate salts in KBr pellets indicate distinctive strong amide absorption at approximately 1620-1655 cm^{-1}, a weaker carbonyl C=O absorption at about 1394-1454 cm^{-1}, a strong hydroxyl OH absorption at about 3400-3450 cm^{-1} and a characteristic strong C-N groups absorption in the range from 1085 to 1165 cm^{-1}. The absorption peaks between 2900 cm^{-1} and 2800 cm^{-1} are characteristic of aliphatic N-H stretching, while those around 1600-1660 cm^{-1} and 1390-1450 exhibit characteristics of amide groups and C=O groups respectively.

2.8.2 *Identification using Nuclear magnetic resonance (NMR) spectroscopy*

NMR spectroscopy is an analytical technique used to ascertain and confirm the structure of organic compounds (Pretsch *et al*., 2009). NMR spectroscopy uses radio-frequencies (usually ranging from 10m to 800 MHz). NMR studies the magnetic properties of the nuclei of atoms to determine the structure of a compound (De Graaf, 2013). Proton (^{1}H) and carbon (^{13}C)-NMR spectroscopies are usually performed to determine γ-PGA homogeneity and degree of esterification (Birrer *et al*., 1994 & Borbely *et al*., 1994). Chemical shifts from resulting NMR spectra are measured relative to known standard.

2.8.3 *Molecular weight determination*

GPC is the most commonly used method for determining the molecular weight of γ-PGA. GPC uses a range of mobile phases and calibrates against standards of diverse molecular weights (Birrer *et al*., 1994). The molecular weight of γ-PGA is important for understanding its function (Sung *et al*., 2005b). The molecular weight of γ-PGA produced by *Bacillus* sp. ranges from 10^5–10^6 Da (Shih & Van, 2001) and would differ depending on the application for which it is required. Molecular weight reduction can be an important step in the production of γ-PGA for a drug delivery application. Different techniques used for this purpose are ultrasonic degradation, alkaline hydrolysis, alteration of medium composition and microbial or enzymatic degradation (Shih & Van, 2001). Ultrasonic degradation in particular was shown to be an effective method to reduce both the molecular weight and the dispersity of naturally produced PGA without disturbing the chemical constitution of the polymer (Pérez-Camero, 1999). In the study of Richard & Margaritis (2006), *in situ* depolymerisation of γ-PGA in the cell-free fermentation broth of *B. subtilis* IFO 3335 was carried out. The molecular weight, when measured using GPC and intrinsic viscosity correlations, reduced from about 4 x 10^6 Da to 5.5 x 10^4 Da over a period of 144 h. As with the study done by Yao *et al*. (2009), the dispersity of γ-PGA decreased as a function of hydrolysis time. Enzymatic degradation seems to be a better method to obtain γ-PGA of the required molecular weight in a controlled fashion.

2.8.4 *Amino acid analysis*

The detection of only glutamic acid in amino acid and TLC analysis expresses the purity of γ-PGA (Shih and Van, 2001). For amino acid analysis of γ-PGA, purified γ-PGA is hydrolysed with 6N HCl at 100 °C for hours (depending on the amount of γ-PGA) in an airtight tube, followed by the removal of residual HCl by evaporation and then the hydrolysed product is dissolved in distilled water before analysing the amino acid contents by TLC (Yokoi *et al*.,

1995). According to Shih *et al*. (2001), purified γ-PGA is hydrolysed with 6N HCl at 110 °C for 24 h in a closed and evacuated tube and analysed using an amino acid analyser. TLC is then carried out on a cellulose plate with solvent systems of butanol–acetic acid–water (3:1:1 w/w) and 96% ethanol–water (63:37 w/w) and amino acids were identified by spraying with 0.2% ninhydrin in acetone (Yokoi *et al*., 1995).

2.9 Applications of poly-γ-glutamic acid

γ-PGA is an important polymer with interesting and unique properties such as its biodegradability, solubility in water, non-toxicity, edibility and viscosity which makes it and its derivatives of high industrial attraction. γ-PGA has many applications across various fields particularly in the treatment of wastewaters laden with heavy metals.

2.9.1 *Water/Wastewater treatment*

γ-PGA comprises of carboxyl groups and as a result have high cation exchange capacity. The amide groups present in the skeleton of γ-PGA are also electron donors, and sometimes both groups together have a synergistic effect (Wang *et al*., 2014). As a result, γ-PGA and its derivatives have been reported to bind several metal ions including Hg^{2+}, Cu^{2+}, Pb^{2+}, Cd^{2+}, Cr^{3+}, and Ni^{2+}.

The adsorption capacity of a novel hybrid gel (ALG-PGA), derived by crosslinking calcium alginate and γ-poly glutamic acid towards rare earth – neodymium (Nd^{3+}) was studied. Results showed that the adsorption reaction is based on a cation-exchange mechanism (Wang *et al*., 2014). A complex of γ-PGA and its cross-linked nanoparticles were examined for the removal of Fe^{3+} from an aqueous environment (Bodnar *et al*., 2013). γ-PGA was reported to bind Fe^{3+} ions as well as form stable particulate complexes. The study showed that linear or cross-linked γ-PGA can be used in nano-membrane technology for effective removal of Fe^{3+} ions and rapid filtration.

Chang *et al*. (2013) investigated the capability of γ-PGA-modified super-paramagnetic iron-oxide nanoparticles (γ-PGA/Fe_3O_4 MNPs) in the removal of heavy metal ions including Cr^{3+}, Cu^{2+}, Pb^{2+}, and Ni^{2+}. γ-PGA/Fe_3O_4 MNPs showed outstanding removal activity for all metals and better removal efficiency than either Fe_3O_4 MNPs or γ-PGA only while overcoming the weaknesses of using either Fe_3O_4 MNPs or γ-PGA on their own. This indicates that γ-PGA has potential applications in potential applications in wastewater treatment.

The removal of toxic lead ions from aqueous solution via a biodegradable nanoparticle-enhanced ultrafiltration technique was also investigated by Hajdu *et al*. (2012). Linear γ-PGA and its cross-linked nanoparticles were used to remove Pb^{2+} ions by forming complexes with the lead ions and then getting rid of them via membrane separation. Results showed that γ-PGA could bind and remove more than 99.8% of the Pb^{2+} ions present in the water through an ultrafiltration method.

γ-PGA (M_w 5.8 x 10^6 Da) produced from *Bacillus subtilis 2063* showed great flocculating activity indicating that γ-PGA can be a potential alternative to conventional synthetic flocculants (known to persist in the environment) in wastewater treatment (Bhunia *et al*., 2012). Similarly, Bajaj and Singhal (2011a) studied the flocculation properties of γ-PGA (M_w 6.2 x 10^6 Da) derived from *B. subtilis* R 23. Results indicated that γ-PGA is an excellent flocculant and can be used in wastewater treatment plants and downstream processing of food and fermentation industries.

Bodnar *et al*. (2008) also investigated the formation of complexes of γ-PGA derived from *B. licheniformis* 9945a with bivalent lead ions. Results showed that the size of the complexes formed depended on both the pH and concentrations of γ-PGA and lead ions. The complex was stable in aqueous media at low, neutral and mild alkaline conditions. Large flocs were formed at low pH values and high concentration of γ-PGA and lead ions. This complex is

thought to have useful water treatment applications due to its biodegradability along with heavy metal binding activity.

γ-PGA was investigated in the removal of basic dyes including Auramine O, Rhodamine B and Safranin O which are harmful to aquatic organisms, animals and in turn humans from dilute wastewater and aqueous solution (Inbaraj *et al*., 2006). Findings showed that γ-PGA (M_w: 9.9 x 10^5 Da) can be used effectively to remove basic dyes from aqueous solution. It was also discovered that 98% of the dye adsorbed on γ-PGA could be recovered at pH 1 which facilitates the reuse of spent γ-PGA. It has also been reported to be effective in the removal of methylene blue and malachite green dyes from aqueous solution (Inbaraj *et al*., 2009). Since γ-PGA is non-toxic and biodegradable, the adsorption system developed could offer eco-friendly solutions to the dye industry.

2.9.2 *Other applications*

A polyelectrolyte complex (PEC) comprising of chitosan as a cationic polyelectrolyte and γ-poly (glutamic acid) (γ-PGA) as an anionic polyelectrolyte was designed by Tsao *et al*. (2011) for wound dressing. Results showed that the chitosan/γ-PGA PECs exhibit adequate moisture content, with γ-PGA reducing the risk of dehydration compared to regular chitosan. Preclinical studies in animal models showed that wounds treated with the chitosan/γ-PGA PECs healed faster than wounds given no treatment. Chitosan/γ-PGA PECs showed excellent suppression of inflammatory cells in contrast to neat chitosan indicating that γ-PGA has anti-inflammatory effect. Keratin development was more assimilated in those wounds treated with chitosan/γ-PGA PECs compared to the neat chitosan or control. Chitosan/γ-PGA PECs were also easily taken off the wound surface causing damage to the newly regenerated tissue after healing suggesting that chitosan/γ-PGA PECs are potential materials for wound dressings.

The effect of γ-PGA on the viability of probiotic bacteria during freeze drying was investigated by Bhat *et al*. (2013). Results showed that 10% γ-PGA was found to protect *Lactobacillus paracasei* significantly better than 10% sucrose which has been previously reported to offer better protection during freeze drying of lactobacilli compared to trehalose and sorbitol (Siaterlis *et al*., 2009) and *nata*, a bacterial cellulose produced by *Acetobacter xylinum* (Jagannath *et al*., 2010).

The effects of γ-PGA on the viscosity, foam stability and emulsifying properties of sponge cake batter at different concentrations (0.05, 0.1, 0.5 g/kg w/w) were explored by Shyu and Sung (2010). The addition of 0.5g/kg γ-PGA to sponge cake batter showed an increase in viscosity, foam stability and emulsion stability. Sponge cake hardness and chewiness were also decreased during storage with the addition of γ-PGA.

γ-PGA was found to increase calcium absorption *in vitro* and *in vivo* and decrease bone loss in humans (Tanimoto *et al*., 2007). γ-PGA increased the bioavailability of calcium by increasing its solubility and intestinal absorption. The increase in calcium solubility was as a result of the inhibition of the production of an insoluble calcium phosphate (Tanimoto *et al*., 2007). Post-menopausal women who were given a single dose of γ-PGA were seen to have better intestinal absorption. Individuals with lower basal absorptive capacity were particularly seen to benefit from γ-PGA.

The applications of γ-PGA in skin care products were explored by Ben-Zur & Goldman (2007). They found that γ-PGA is a good hydrophilic humectant and has the ability to increase the production of natural moisturizing factors like urocanic acid, pyrrolidone carboxylic acid and lactic acid compared to hyaluronic acid and soluble collagen. γ-PGA has also been shown to enhance the elasticity of the skin more than collagen and hyaluronic as well as refresh and nourish the skin making it smoother and desirable (Ben-Zur and Goldman, 2007).

Sung *et al*. (2005a) successfully utilized γ-PGA as an active ingredient in a hyaluronidase inhibitor. Hyaluronidase is an enzyme that degrades hyaluronic acid present in the skin dermis. The composition was tested on fifty women (age group between 30 and 50) and results showed that skin elasticity was maintained by inhibiting the activity of hyaluronidase and that allergenic reaction was reduced by inhibiting the permeability of inflammatory cells.

Hu *et al.* (2008) studied the possibility of using γ-PGA to induce protein crystallization in easily crystallizable proteins such as lysozyme, glucose isomerase and xylanase. Both low molecular weight (LM_w) γ- PGA (Na^+ salt – M_w: 2 x 10^5 - 4 x 10^5 Da) and high molecular weight (HM_w) (Na^+ salt – M_w: >1x 10^6 Da) were tested and results showed that γ-PGA could indeed induce precipitation in proteins as additives or stand-alone precipitants.

The number of potential applications for γ-PGA is vast and still increasing. **Table 2.2** attempts to summarise the diversity of γ-PGA application within the range of application areas.

Table 2.2: Applications of γ-PGA and its derivatives

Field	Application type	Functions	References
Wastewater Treatment	Biopolymer flocculant	γ-PGA produced by *B. subtilis* R 23 showed a high flocculating activity that can be further enhanced by the addition of cations.	(Bajaj and Singhal, 2011a)
		Efficient flocculation of various organic and inorganic compounds was demonstrated by γ-PGA produced by *B. licheniformis* CCRC 12826.	(Shih *et al*., 2001)
	Heavy metal removal	γ-PGA covalently incorporated into microfiltration membranes via attached to their membrane pore surfaces exhibited super-high heavy metals sorption ability.	(Bhattacharyya *et al*., 1998)
		γ-PGA binds and efficiently removes > 99.8% of lead ions from water via a suitable, low-pressure ultrafiltration technique.	(Hajdu *et al*., 2012)
	Dye removal	γ-PGA (molecular weight: 9.9 x 10^5 Da) can be used effectively to remove basic dyes from aqueous solution. It was found that 98% of the dye adsorbed on γ-PGA could be recovered at pH 1 which facilitates the reuse of spent γ-PGA	(Inbaraj *et al*., 2006)
Medicine	Metal chelator	γ-PGA coated super paramagnetic iron oxide nanoparticles demonstrated high heavy metal removal efficiency from simulated gastrointestinal fluid and a meal solution.	(Inbaraj and Chen, 2012)
	Drug carrier/deliverer	γ-PGA with covalently attached Cisplatin (cis-dichlorodiammineplatinum (II), CDDP] was shown to reduce the toxicity of CDDP while efficiently decreasing the tumour size of xenografted human breast in nude mice as well as lengthen the survival of nude mice grafted with Bcap-37 tumour cells.	(Ye *et al*., 2006)
		A macromolecular conjugate of paclitaxel and γ-$_L$-PGA called Paclitaxel poliglumex (PPX) exhibited outstanding benefits over conventional paclitaxel. PPX was accumulated in tumour tissue, where it gradually discharges the active agent paclitaxel.	(Singer, 2005)
	Tissue engineering	A γ-PGA/chitosan composite biomaterial has demonstrated potential application in tissue engineering as it is more hydrophilic and cytocompatible than convention chitosan matrices.	(Hsieh *et al*., 2005)
		A polyelectrolyte complex of chitosan and γ-PGA demonstrated potential application in wound dressing. The complex presented sufficient moisture content and showed good mechanical properties which allow dressing to be easily removed from the wound surface without destroying renewed tissues.	(Tsao *et al*., 2011)

	Biological adhesives	A mixture of gelatin and γ-$_{L}$-PGA aqueous solution which resulted in the formation of a hydrogel in the presence of water-soluble carbodiimide (WSC), demonstrated better lung adhesion and air leak sealing than conventional fibrin glue.	(Otani *et al*., 1999)
Food industry	Food supplement/ osteoporosis-preventing agent	Natto mucilage containing *γ*-PGA greatly improved Calcium solubility *in vitro* and *in vivo* in rats as well as the calcium content of their bones.	(Tanimoto *et al*., 2001 and Ho *et al*., 2009)
	Texture enhancer	It has been established that the addition of *γ*-PGA to wheat bread reduces its hardness through storage. *γ*-PGA also enhances the rheological and thermal properties of wheat dough. *γ*-PGA was demonstrated to improve the texture of sponge cake.	(Shyu *et al*., 2008 and Shyu and Sung, 2010)
	Oil reducing agent	*γ*-PGA has been proven to reduce oil uptake during deep-fat frying. *γ*-PGA added doughnut showed a 5-fold increase in oil reduction compared to normal doughnut. The overall appearance and taste of the product was better than that of normal doughnut.	(Lim *et al*., 2012)
	Cryoprotectant	*γ*-PGAs with molecular weights lower than 20,000Da have proven to have higher antifreeze activities than high antifreeze agents like glucose and they do not interfere with the taste of foods.	(Mitsuiki *et al*., 1998)
		γ-PGAs with molecular weight of 257,000 Da has been used as a cryoprotectant for probiotic bacteria to improve their survival during production.	(Bhat *et al*., 2013)
	Bitterness relieving agent	γ-PGA has also been used as a bitterness relieving agent	(Sakai *et al*., 2000)
Cosmetics	Moisturizer	*γ*-PGA has been demonstrated to improve the qualities of skin and hair – care products such as exfoliating, moisturizing and removing wrinkles.	(Ben-Zur and Goldman, 2007)
Agriculture	Bio-control agents and/or fertilizer synergists	Lipopeptides and *γ*-PGA produced by *B. subtilis* B6-1using soybean and sweet potato residues adequately repressed cucumber wilts, increased nutrient consumption as well as increased the growth of cucumber seedlings	(Wang *et al*., 2008)
Others	Biodegradable plastics	Biodegradable plastics comprising of α-radiation crosslinked PGA or a PGA salt as the major constituent have been reported.	(Tsutomu and Makoto, 2002)
		Esterified γ-PGA produced by *Bacillus subtilis* F2-01 was discovered to be a good thermoplastic. Ester derivatives of γ-PGA have also demonstrated the capability to form biodegradable fibres and films thereby eliminating non-biodegradable polymers.	(Kubota *et al*., 1995 and Shih and Wu, 2009)

Gene delivery	A γ-PGA complex pDNA/PEI/γ-PGA was developed and used for brilliant gene delivery with very high transgene capability and low toxicity	(Kurosaki *et al*., 2009)
Antibacterial activity	Sodium and calcium salts of Poly (γ-glutamic acid) NaPGA and CaPGA - coated magnetite nanoparticles (MNPs) demonstrated antibacterial activity against Salmonella enteritidis SE 01 compared to commercial antibiotics linezolid and cefaclor and are cytocompatible. NaPGA-MNPs was also active against Escherichia coli ATCC 8739 and Staphylococcus aureus ATCC 10832, while CaPGA-MNPs was active against Escherichia coli O157:H7 TWC 01	(Inbaraj *et al*., 2011)
Calcium absorption	Administration of γ-PGA increased calcium absorption in the intestine in post-menopausal women by inhibition of formation of an insoluble calcium complex with phosphate. Can be potentially used for treatment of bone disorders.	(Tanimoto *et al.*, 2007)
Inhibition of influenza virus	γ-PGA based glycopolymers (used to inhibit influenza virus) showed higher solubility in water & heat stability and lower toxicity & immunogenicity compared to glycopolymers without γ-PGA	(Ogata *et al.*, 2009)
Glucose sensor	Needle-type glucose sensors prepared by covalent immobilization of glucose oxidase on γ-PGA film. Increased stability of the electrode to a month.	(Yasuzawa *et al.*, 2011)
Treatment of xerostomi (dry mouth)	γ-PGA promoted salivary secretion and produces moisturizing effect. Solved problems associated with sticky displeasure of the mouth, difficulties in speaking, bad breath, dental caries, periodontal disease and mucosal infectious disease.	(Uotani *et al.*, 2011)

2.10 Aims of this study

The aims of this research are:

- To investigate the production of poly-gamma-glutamic acid (γ-PGA) using five *Bacillus* strains
- To optimize the growth media by control of substrate concentration and environmental conditions in order to obtain high molecular weight (HMW) γ-PGA
- To analyse the γ-PGA produced by each bacterium based on yield, form (salt or acid), molecular weight and crystallinity.
- To identify and select the suitable bacterium and medium for producing HMW γ-PGA
- To use the selected bacterium and medium for the production of HMW γ-PGA
- To assess the effectiveness of HMW γ-PGA in the clean-up of waters contaminated with Cu^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+} and Ag^{+}.

3.0 MATERIALS AND METHODS

3.1 Production of poly-gamma-glutamic acid (γ-PGA)

3.1.1 Bacterial strains

The five *Bacillus* strains (*Bacillus licheniformis* 1525, *Bacillus licheniformis* NCTC 6816 *Bacillus licheniformis* ATCC 9945a, *Bacillus licheniformis* ATCC 9945 (also known as *Bacillus licheniformis* CCRC 12826) and *Bacillus subtilis* (natto) ATCC 15245 (also known as *Bacillus natto* Sawamura) used in this study were obtained from the National Collection of Industrial and Marine Bacteria (NCIMB). All stock cultures were freeze-dried and stored at -20 ^{o}C to preserve the bacterial strains. Prior to use, frozen cells were aseptically resuscitated in TSB and grown on general purpose agar at 37 ^{o}C for 24-48 hours.

3.1.2 Growth Media

Tryptone soy agar TSA made of: tryptone casein digest 1.5%, soy peptone 0.5%, agar 1.2%, K_2HPO_4 0.25% and D-glucose 0.25%, tryptone soy broth (TSB) made of: tryptone casein digest 1.7%, soy peptone 0.3%, NaCl 0.5%, K_2HPO_4 0.25% and D-glucose 0.25% and ¼ strength Ringer's solution all obtained from Lab M, UK were prepared according to manufacturer's instructions.

The fermentation media employed in the production of γ-PGA were GS medium (Sung et al., 2005b), medium C (this study) and medium E (Leonard et al., 1958) . Citric acid – an important component of medium E is known to be the best precursor for γ-PGA production (Du *et al*., 2005). Therefore medium E was used to ascertain if the strains used in this study could synthesize γ-PGA in the presence of the precursor. GS medium was on the other hand employed to determine if γ-PGA could be synthesized in the absence of precursors. GS medium was composed of (g/L) L-glutamic acid (20) [Sigma-Aldrich], sucrose (50) [Fisher Scientific], KH_2PO_4 (2.7) [Sigma-Aldrich], Na_2HPO_4 (4.2) [Acros Organics], NaCl

(5) [Fisher Scientific], $MgSO_4.7H_2O$ (5) [Sigma-Aldrich] and 1 ml of Murashige-Skoog vitamin solution [Sigma-Aldrich] which was aseptically added to the medium after autoclaving just before inoculation. The pH of the medium was initially adjusted to 5.0 before autoclaving using 3M NaOH [Fisher Scientific] and then finally adjusted aseptically to 6.8 after autoclaving. A new medium - medium C is an optimized GS medium with citric acid precursor. Medium C comprised of (g/L) L-glutamic acid (20) [Sigma-Aldrich], citric acid (12) [Sigma-Aldrich], sucrose (50) [Fisher Scientific], KH_2PO_4 (2.7) [Fisher Scientific], Na_2HPO_4 (4.2) [Fisher Scientific], NaCl (5) [Fisher Scientific], $MgSO_4.7H_2O$ (5) [Sigma-Aldrich] and 1 ml of Murashige-Skoog vitamin solution [Sigma-Aldrich] which was aseptically added to the medium after autoclaving just before inoculation. Medium E contained (g/L) L-glutamic acid (20) [Sigma-Aldrich], glycerol (80) [Sigma-Aldrich], citric acid (12) [Sigma-Aldrich], NH_4Cl (7) [Riedel-de Haën], $MgSO_4.7H_2O$ (0.5) [Sigma-Aldrich], $FeCl_3.6H_2O$ (0.2) [Sigma-Aldrich], K_2HPO_4 (0.5) [Sigma-Aldrich], $CaCl_2.2H_2O$ (0.15) [Fisher Scientific] and $MnSO_4.H_2O$ (0.2) [Sigma-Aldrich]. The pH was adjusted to 7.2 using 3M NaOH [Fisher Scientific] and 1M HCl. All media were sterilized by autoclaving at 121°C for 20 minutes at 15p.s.i. with the exception of sucrose and Murashige-Skoog vitamin solution which were sterilized at 110°C for 20 minutes at 10 p.s.i. in a pressure cooker and by filtration respectively. All other reagents used in this study were of approved grade.

3.1.3 Inoculum preparation

Highly mucoid colonies of each bacterial strain on the plate were taken and inoculated into 100 ml of TSB in 250 ml conical flask using a sterile inoculation loop and aerobically incubated at 37^oC overnight with shaking at 150 rpm. The inoculum reached a population of about 7 log CFU/ml.

3.1.4 Production of γ-PGA in shake flask cultures

For flask cultures, all fermentation was carried out in 500 ml conical flasks which are corked with foam bungs and wrapped up with aluminium foil. 5% inoculum level was inoculated into 250ml of production medium and cultivated aerobically in a rotary incubator (Innova 43, USA) at 37^{o}C or 50^{o}C and 150 rpm for 96 hours. All flask cultures were carried out in triplicates. γ-PGA production time usually depends on the bacterial strain and medium utilised in the production. According to previous researches, γ-PGA production occurs in the late exponential and stationary phase of the growth cycle (Bajaj and Singhal, 2011b; Buescher and Margaritis, 2007 and Shi *et al*., 2007). Samples were periodically taken from the flask cultures at time 0, 2, 4, 24, 48, 72 and 96 hours and used in the determination of pH, cell growth and/or γ-PGA production.

3.1.5 Bacterial growth determination

Bacterial growth was determined by cell viability using plate count. Samples taken at time intervals from the fermentation culture were serially diluted and plated out onto TSA using the Miles and Misra and spread plate techniques.

For plate count, 8 tubes of 4.5 ml ringer solution were labelled from 10^{-1} to 10^{-8} to carry out a tenfold serial dilution. 0.5 ml (500 µl) of sample was aseptically taken from the growing culture and transferred into the first tube of 4.5 ml of sterile ringer solution (stock) using a sterile Finn pipette and aspirated with a vortex mixer. 0.5 ml of sample was drawn up from the stock solution using a fresh pipette tip and aseptically transferred into the next test tube (dilution 10^{-2}) and vortexed. In the same manner; the dilution was repeated based on the number of dilutions to be plated out. At incubation time 0, 2 and 4 hours, dilutions 10^{-3} to 10^{-6} were plated out, but as the incubation time increases, the viscosity of the culture broth increased which indicated increase in growth and thereby increased the number of dilutions to be plated out for incubation time 24, 48, 72 and 96 hours to dilutions 10^{-3} to 10^{-8}.

For Miles and Misra method, starting from the lowest dilution, 20 µl of sample was drawn up from each test tube labelled 10^{-3} to 10^{-8} using a Finn pipette and transferred onto the centre of each sector on TSA plates divided into 8 sectors and labelled accordingly with name, time and dilutions.

For spread plate technique, also starting from the lowest dilution, 100 µl of sample was taken from each test tube labelled 10^{-3} to 10^{-8} using a Finn pipette and transferred onto the centre of each TSA plates correctly labelled with name, time and dilution.

Each dilution was plated in triplicates and plates were incubated at 25^{o}C overnight. The number of colonies on each plate was counted. For the Miles and Misra method, plates with colony count of 3 to 30 were counted and for the later, plates with colony count of 30 to 300 colonies were counted and recorded. The average number of colonies from the triplicates was used to deduce the colony forming units per 1 ml of sample (CFU/ml) using the following equations:

Miles and Misra: CFU/ml = average colony count x 50 x dilution plated.

Spread plate: CFU/ml = average colony count x 10 x dilution plated

The colony forming units per ml (CFU/ml) values for all replicates (n=3) were converted to Log_{10} values and the average values obtained were used to devise a fermentation growth curve. Each experiment was repeated three times and analysed statistically.

3.1.6 Extraction and recovery of γ-PGA

Highly viscous fermentation broths obtained after cultivation for 96 hours were transferred from the conical flasks into 500 ml centrifuge tubes. Cells were then separated from broth which contains the γ-PGA by centrifugation at 17,001 x *g* and 9600 rpm for 30 minutes at 4^{o}C using Sigma centrifuge (Steinheim, Germany). The residual cells were inactivated using trigene before appropriate disposal. The supernatants were transferred into shott bottles where 4 volumes of cold ethanol (95%) were poured into the supernatant, it was gently stirred and left undisturbed for 72 hours in the cold room to allow precipitation, after which the waste ethanol was decanted to obtain the resultant precipitates. The precipitates were centrifuged at 17,001 x *g* and 9600 rpm for 30 minutes to accumulate the crude γ-PGA and remove all waste ethanol. The resultant residues from the centrifugation were dissolved in distilled water using a magnetic stirrer (Progen Scientific Limited, UK). The dissolved precipitates were transferred into Spectra/Por® Dialysis Membrane (MWCO: 10,000) tubes and placed in a beaker of deionised water at room temperature for 24 hours. This technique is required to remove impurities lower molecular weights lower than 10,000 Da. The recovered solutions were then transferred into 500 ml round bottom flasks for lyophilisation and gently rotated in cold ethanol and dry ice for few minutes to freeze the polymer in form of a thin layer around the flask to provide a large surface area for drying. The frozen polymer was lyophilized using a freeze dryer (Edward Modulya, UK) at 0.4 atm. and -20^{o}C for 72 hours after which the dried γ-PGA samples were collected, weighed to determine the yield (g/l) and stored in a desiccator for further analysis.

3.1.7 Identification of γ-PGA

3.1.7.1 Fourier transform infra-red (FT-IR) spectroscopy

The need to identify the recovered polymer as γ-PGA was necessary for further analysis and application. Fourier Transform Infra-Red Spectroscopy (FT-IR) technique which uses an infrared absorption spectrum was used in the identification of this biopolymer. FT-IR is a non-destructive, quick method that gives accurate results without any external calibration.

For γ-PGA identification, a Genesis II FTIR™ spectrometer (SEMSIR, UK) was used. Approximately 0.5g of finely ground γ-PGA samples was placed onto the scanner of the spectrometer, and the samples were loaded by using a small metal rod to apply pressure to the sample. A background scan was first carried out before the sample was scanned to obtain spectra. The FT-IR spectrometer uses software called WINFIRST which scanned the samples and produced resulting Infra-Red (IR) spectra with different peaks. The resulting spectrum usually represents the molecular absorption and transmission thereby producing a molecular fingerprint of the sample. Each spectrum is unique to its own molecular structure and each peak in a spectrum represents a specific bond in the compound. The peaks of the spectra obtained from the different γ-PGA samples synthesized during the course of this project work were compared with the standard peaks of commercial samples of γ-PGA for peaks identification.

According to Ho *et al*. (2006), the infrared spectra of γ-PGA (free acid form) and γ-polyglutamate salts in KBr pellets indicate distinctive strong amide absorption at about 1620-1655 cm^{-1}, a weaker carbonyl C=O absorption at about 1394-1454 cm^{-1}, a strong hydroxyl OH absorption at approximately 3400-3450 cm^{-1} and a characteristic strong C-N groups absorption in the range from 1085 to 1165 cm^{-1}. The absorption peaks between 2900 cm^{-1} and 2800 cm^{-1} were characteristic of aliphatic N-H stretching, while those

around 1600-1660 cm^{-1} and 1390-1450 exhibited characteristics of amide groups and C=O groups respectively.

Table 3.1 gives a summary of the data of FT-IR absorption peaks for γ-PGA and γ-polyglutamate salts as reported by Ho *et al*. (2006).

Table 3.1: Summarised data of FT-IR absorption peaks for γ-PGA and γ-polyglutamate salts.

Chemical bonds	Absorption peak
Amide I, N-H	1622-1643
Amide II, Stretch	1585
C=O Stretch	1739
C=O Symmetric Stretch	1402-1454
C-N Stretch	1089-1140
N-H Bending	616-707
O-H Stretch	3402-3449

Source: Ho *et al*., 2006.

3.1.7.2 Nuclear Magnetic Resonance (NMR) Spectroscopy

The structure and purity of γ-PGA was characterized by proton nuclear magnetic resonance (^{1}H NMR) and carbon nuclear magnetic resonance (^{13}C NMR). Both ^{1}H and ^{13}C NMR spectra were recorded with the use of a Bruker Avance II spectrometer operating at 600MHz with Bruker TOPSPIN 2.0 Software. ^{1}H NMR spectra were obtained with 64 scans, 2.65s acquisition time and 11 μs pulse width while ^{13}C NMR spectra were obtained with 76,800 scans, 0.9088s acquisition time and 9.40 μs pulse width. ^{1}H NMR and ^{13}C NMR spectra were run at room temperature using D_2O as the solvent and tetramethylsilane (TMS) as internal standard. The chemical shifts are represented in parts per million (ppm) based on the signal for the standard.

3.1.8 Analysis of γ-PGA using inductively coupled plasma spectrometry (ICP)

Produced γ-PGA could be in different forms. It could be in form of a salt or free acid or both (free acid form and salt form). Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) was used to separate and quantify γ-PGA components (free acid form and salt form) according to their mass. ICP-AES is an analytical method used in the separation of a compound into elemental parts. It uses inductively coupled plasma (ICP) which generates excited atoms and ions that emit electromagnetic radiation at wavelength specific to a particular element. The intensity of the electromagnetic radiation emitted at a particular wavelength is proportional to the concentration of that element within the sample.

Prior to ICP-AES analysis using SPECTRO CIROS^{CCD} (Germany), dissociation of the γ-PGA sample into its component element was carried out. About 0.25 g of dried γ-PGA sample was weighed out into plastic vials; 6 ml of nitric acid (HNO_3) and 1 ml of hydrogen peroxide (H_2O_2) were added to the samples in the vials. The volume was made up to 25ml and digested using an ETHOS 900 Microwave Labstation (Milestone Microwave Laboratory System) using a previously fed computer program. The final volume was made up to 50 ml after digestion for ICP-AES analysis. The sample was aspirated using a sipper probe connected to a peristaltic pump and then scanned for a range of elements. The concentration of each element in the sample was represented in [mg/l]. The concentration of the elements determined after the analysis represents the salt form of γ-PGA of the particular element. The concentration of the salt form of γ-PGA of a particular element in percentage was determined using the calculation below:

$$\%\ \text{content of salt form of } \gamma\text{-PGA} = \frac{(Mw\ of\ \gamma{-}PGA\ salt) * (Concentration\ of\ element\ in\ ppm)}{(Mw\ of\ element) * 10^6} * \text{D.F} * 100$$

Where: M_w is the molecular weight, D.F. is the dilution factor

3.1.9 Molecular weight of γ-PGA

Determination of the molecular weight of γ-PGA is very important for γ-PGA application. The molecular weight (M_w) of γ-PGA alongside its molecular number and (M_n) and dispersity index (*Đ*) was determined using conventional aqueous based gel permeation chromatography (GPC). *Đ* is the measure of the wideness of molecular weight distribution in a given polymer sample and is determined using the equation: $Đ = \frac{M_W}{M_n} \geq 1$. The dispersity index is a representative of the broadness of the molecular weight i.e. the nearer the dispersity is to unity, the more uniform the molecular weight of the polymer. GPC separates analytes according to their sizes by allowing them (the analytes) pass through porous beads packed in a column. The smaller the analytes are, the easier it is for them to enter the pores thereby spending more time within the pores and increasing their retention time as opposed to larger analytes.

Molecular weight analysis of γ-PGA was carried out at Smithers Rapra in Shrewsbury, UK. A Viscotek Triple Detector Array TDA301 with PL aquagel-OH guard plus 2 * PL aquagel-OH MIXED-H was used for the analysis. γ-PGA is insoluble in organic solvents and as such they cannot be used as eluent. Therefore, 0.2M $NaNO_3$, 0.01M NaH2PO4 (pH 7) was used as eluent with a flow rate of 1.0 ml/min at 30^oC and a refractive index detector (with differential pressure and light scattering). The data was collected and analysed using Malvern/Viscotek 'OmniSec' software. Samples were prepared by adding 10 ml of eluent to about 20 mg of γ-PGA samples. The solutions were left overnight to dissolve completely and warmed up at 50^oC for 30 minutes the following day. The solutions were then filtered through a 0.45 µm PVDF directly into auto sampler vials after cooling. A range of sodium polyacrylate calibrants obtained from Agilent/Polymer Laboratories was used to calibrate the GPC system used for this work.

3.1.10 Crystallinity of γ-PGA

A polymer is usually made up of both crystalline and amorphous phases arranged randomly. An understanding of polymer crystallinity is very crucial because the mechanical properties of crystalline polymers differ from those of amorphous polymers. Polymer crystallinity is also important for the selection of a polymer for an application since it influences its properties such as thickness, tensile strength, stiffness, melting point and solubility. Amorphous polymers are those with no substantial degree of crystallization, where the polymer chains have no well-defined order in either the solid or liquid states while crystalline polymers are those with substantial degree of crystallization, where polymer molecules are closely packed and in organised order.

Crystallinity of γ-PGA was determined using powder X-Ray Diffraction (XRD) analysis. XRD is a non-destructive analytical procedure where atomic planes of a crystal make a beam of X-rays scatter at specific angles from each set of lattice planes in a sample. The intensities of the peaks are determined by the arrangement of atoms within the lattice. Regularly distributed atoms within the sample reflect the x-ray beam constructively and results in sharp narrow peaks representing a crystalline sample while curves emerging as a result of randomly occurring atoms represents an amorphous sample. A PANalytical empyrean X-ray diffractometer was used to obtain high quality diffraction data of γ-PGA samples. About 0.2 g of pure γ-PGA was obtained and ground into a fine powder. The fine powder was then poured into a sample holder using a thin spatula until the sample holder was completely filled. It is important to gently tap the sample holder on a table to compact the powder in the holder. Powder was then pressed onto the sample holder using a glass slide to ensure that the top of the sample is in the same plane as the top of the sample holder.

3.1.11 Toxigenic analysis

Toxigenic analysis of the *Bacillus* strains used in the production of γ-PGA is essential for safe application of γ-PGA. Hence, the presence (not expression) of major enterotoxins and virulence factors in *Bacillus subtilis* (natto) ATCC 15245, *Bacillus licheniformis* ATCC 9945a and *Bacillus licheniformis* ATCC 9945 were studied and compared to *Bacillus cereus* as a positive control (Matarante *et al*., 2004 and Bhat *et al*., 2013). The genes encoding various enterotoxins and enzymes investigated includes haemolysin BL enterotoxin (hbl D/A), non-haemolytic enterotoxin (nheB), sphingomyelinase (sph), phosphatidylcholine (piplc), *Bacillus cereus* enterotoxin T (bceT) and enterotoxin FM (entFM). Two housekeeping genes - *Bacillus subtilis* phosphofructokinase A (pfkA) and *Bacillus licheniformis* pfkA were employed as positive controls. PCR analysis was used to investigate presence of these toxins.

DNA extraction is important for PCR analysis. Therefore, DNA was first isolated and purified before the analysis. A single colony of all three strains plus *B. cereus* were inoculated in 5 ml of TSB and incubated at 30°C overnight with shaking at 150 rpm. Cells were collected by centrifugation at 1,000 × *g* for 5 min and washed with sterile water. GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, UK) was used for DNA isolation based on manufacturer's instructions. The amount of DNA extracted was then determined using a Nanodrop2000 Spectrophotometer (Thermo Scientific, UK).

For PCR analysis, primers were purchased from Sigma-Aldrich, UK (**Table 3.2**). The gene sequence of *Bacillus licheniformis* pfkA was obtained from the nucleotide collection of the National Centre for Biotechnology Information (NCBI) and NCBI Primer-Blast was used to design the primers for the house keeping gene. PCR analysis was carried out as reported by Bhat *et al*. 2013. PCR amplification was performed in an MJ Research PTC-200 Peltier Thermal Cycler. Taq polymerase, deoxynucleotide triphosphates and DNA molecular

weight markers were obtained from Roche, UK. The amplified fragments were electrophoretically separated on a 2% agarose gel.

Physiological studies were carried out on the strains to evaluate their haemolytic and lecithinase potential while using *Bacillus cereus* as a positive control. For haemolytic activity, 5µl of cells grown in TSB at 37˚C overnight were inoculated on 5% horse blood agar plates and incubated aerobically at 30°C for 48 hours. Similarly, 5µl of cells grown in nutrient broth at 37˚C for 24 hours were inoculated on 8% egg yolk emulsion supplemented nutrient agar and incubated aerobically at 30°C for 24 hours to determine lecithinase (phosphatidylinositol-specific phospholipase C) activity (Matarante *et al*., 2004 and Bhat *et al*., 2013).

Table 3.2: PCR primers used and virulence factors

Target gene	Primer name	Primer sequence (5'-3')	Amplicon size (bp)	Reference
hbl-D/A	hblD-fhblA-r	GGAGCGGTCGTTATTGTTGTGCCGTATC TCCATTGTTCGT	623	(Matarante *et al*., 2004 and Bhat *et al*., 2013)
nheB	nheB 1500 S nheB 2269 A	CTATCAGCACTTATGGCAG ACTCCTAGCGGTGTTCC	769	(Matarante *et al*., 2004 and Bhat *et al*., 2013)
bceT	ETF ETR	TTACATTACCAGGACGTGCTT TGTTTGTGATTGTAATTCAGG	428	(Matarante *et al*., 2004 and Bhat *et al*., 2013)
entFM	EntA EntB	ATGAAAAAAGTAATTTGCAGG TTAGTATGCTTTTGTGTAACC	1269	(Matarante *et al*., 2004 and Bhat *et al*., 2013)
sph	Ph1 Ph2	CGTGCCGATTTAATTGGGGC CAATGTTTTAAACATGGATGCG	558	(Matarante *et al*., 2004 and Bhat *et al*., 2013)
piplc	PC105 PC106	CGCTATCAATGGACCATGG GGACTATTCCATGCTGTACC	569	(Matarante *et al*., 2004 and Bhat *et al*., 2013)
B. subtilis pfkA	pfkA-F pfkA-R	CCATCAGCTAAACCAGCC CGCGGTGGTACGAAATTA	370	(Matarante *et al*., 2004 and Bhat *et al*., 2013)
B. licheniformis pfkA	pfkA-F pfkA-R	AACGTCCGCCTTTTCCTTCA ATGCCGGGGACATTGCTTTA	333	Constructed for this study

3.2 METAL SORPTION

3.2.1 Preparation of sorbent

The γ-PGA used was produced from *Bacillus subtilis* (natto) ATCC 15245 via batch fermentation (this study). The molecular weight of γ-PGA as determined by GPC was 1,650, 000 Da.

3.2.2 Metal salts and reagents for sorption experiments

To study the metal ions sorption capacity of γ-PGA, five (5) metal salts of analytical grade were used. These include four (4) divalent metal salts [copper sulphate ($CuSO_4.5H_2O$) [BDH chemicals], zinc chloride ($ZnSO_4.7H_2O$) [BDH chemicals], nickel chloride ($NiCl_2.6H_2O$) [BDH chemicals] and cadmium nitrate tetrahydrate ($Cd(NO_3)_2.4H_2O$) [Acros Organics] and one (1) monovalent metal salt [silver nitrate ($AgNO_3$) [Fisher Scientific]. Hydrochloric acid (HCl) and/or sodium hydroxide (NaOH) were used for pH adjustment.

3.2.3 Sorption experiments

The metal ions sorption experiments were investigated in batch modes and at room temperature (25^{o}C/298 K). 50 mg/l of Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+} and Ag^{+} solutions were prepared by dissolving their respective salts in distilled deionised water. A synthetic solution (50 mg/l) containing all four (4) divalent metal ions was also prepared and used for experimental studies. 100ml of each (50 mg/l) individual metal ion and mixed metal ions solution were transferred into 250 ml Erlenmeyer flasks and know volumes of γ-PGA solution were added to the flasks before adjusting the solutions to required pH. The flasks were sealed with paraffin paper to prevent evaporation and the solutions were mixed on magnetic stirrers at 150 rpm for 12 hours for equilibrium conditions to be attained. The equilibrium period of 12 hours was adopted in order to allow for complete utilization of the γ-PGA added. Samples were then centrifuged at 7200 x *g* for 15 minutes (Yao *et al*., 2006)

to enable the separation of flocs formed as a result of γ-PGA-metal precipitation from the solution and the supernatants were analysed for metal concentration using ICP-AES.

3.2.4 Effect of γ-PGA concentration on metal ion adsorption

The influence of γ-PGA concentration on metal adsorption was investigated at different γ-PGA concentration (0.1, 0.2, 0.25, 0.4, 0.5, 0.6, 0.8 and 1.0 g/l) with metal ion(s) concentration at 50 mg/l and agitation (150 rpm) without pH adjustment for 12 hours. Samples were collected afterwards and analysed for metal concentration as stated above.

3.2.5 Effect of pH on metal ion adsorption

To study the effect of pH on metal adsorption, 50 mg/l metal ion(s) solutions were mixed with γ-PGA (1.0 g/l) and adjusted to different pH values. The experiments were carried out based on the sorption conditions presented above. The sorption tests for each metal ion were investigated at pH values below their maximum solubilities in order to prevent the formation of metal hydroxides (Yao *et al*., 2007). Hence, experiments were performed at pH ranging from 1-8 depending on the metal ion. The maximum pH values chosen for the adsorption of Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+} and Ag^{+} were 6.0, 7.0, 7.5, 8.0 and 7.5 respectively in order to prevent the precipitation of their metal hydroxide. The solutions' pH were monitored and adjusted when necessary during the experiment. Samples were analysed as described above.

3.2.6 Effect of competing heavy metal ions on individual metal ion adsorption

The effect of mixture of heavy metal ions (Cu^{2+}, Zn^{2+}, Ni^{2+} and Cd^{2+}) on the adsorption of individual metal ion by γ-PGA was investigated by mixing 50 mg/l of Cu^{2+}, Zn^{2+}, Ni^{2+} and Cd^{2+} solutions all together with 1.0 g/l of γ-PGA and adjusted to pH 5.5 to avoid precipitation of metal hydroxide for 12 hours. Ag^{+} was excluded from the solution as its addition resulted in the formation of Silver (I) oxide.

3.2.7 Effect of molecular weight on metal ion adsorption

The influence of molecular weight of γ-PGA on the adsorption of heavy metal ion was assessed using a LMW (257,000 Da) and a HMW (1,650,000 Da) γ-PGA. 50 mg/l of Cu^{2+} was mixed with different concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 g/l) of LMW γ-PGA and HMW γ-PGA at room temperature (25^{o}C/298 K) and 150 rpm for 12 hours. Samples were subsequently analysed for metal concentration as earlier described.

3.2.8 Sorption isotherms

For sorption study, γ-PGA concentrations of 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 g/l were chosen after numerous experimental studies to reflect the metal ions' adsorption capacity of γ-PGA in a non-pH controlled system since the increase in dose of γ-PGA was more effective on metal adsorption in a non-pH control system than in a pH controlled system in this study. After equilibrium (12h), the flocs formed were removed by centrifugation and the supernatants were analysed for metal concentration using ICP. All experiments were carried out in triplicates and the average values were taken for data analysis. The amount of metal adsorbed at a particular pH and γ-PGA concentrations was calculated from the mass balance equation below:

$$q = \frac{V}{m}(C_0 - C_e) \quad (1)$$

where q is the amount of metal ion adsorbed per gram of γ-PGA (mg/g), V is the volume of the metal solution (l), m is the amount of γ-PGA (g) and C_o and C_e are the initial and equilibrium metal concentrations (mg/l) respectively (Yao *et al.*, 2007 and Inbaraj *et al.*, 2006). The amounts of metal adsorbed at different pH values and γ-PGA concentrations were also expressed as percentage removal using equation 2 below:

$$R = \frac{(C_0 - C_e)}{C_0} \times 100 \quad (2)$$

where R is the percentage of metal removed (%). The amount of metal ion adsorbed per gram of γ-PGA (mg/g) was plotted as a function of the final metal concentration (Inbaraj *et al*., 2006). Adsorption isotherm data was modelled using both linear and/or non-linear methods of isotherm equations. A non-linear regression method involving Marquardt-Levenberg algorithm was employed to fit three isotherm models including the Langmuir, Freundlich and Redlich-Peterson model using a software called ORIGINLAB. The models can be expressed in linear and/or non-linear forms as detailed in **Table 3.3**.

Table 3.3: Isotherm model equations including x and y values for linear plots

Sorption model	Non-linear form	Linear form	References
Langmuir	$q = \frac{q_{max} K_L C_e}{1 + K_L C_e}$	$\frac{1}{q} = \frac{1}{q_{max}} + \frac{1}{q_{max} K_L} * \frac{1}{C_e}$ $y = \frac{1}{q} \quad x = \frac{1}{C_e}$ $\frac{C_e}{q} = \frac{1}{q_{max} K_L} + \frac{C_e}{q_{max}}$ $y = \frac{C_e}{q} \quad x = C_e$	Bhatt *et al*., 2012, Atulegwu-Patrick *et al*., 2014 and Krishna, 2014
Freundlich	$q = K_F C_e^{1/n_F}$	$\text{Log}\,(q) = \text{Log}\ (K_F) + \frac{1}{n_F}\text{Log}\,(C_e)$ $y = \text{Log}\,(q) \quad x = \text{Log}\,(C_e)$	Bhatt *et al*., 2012 and Atulegwu-Patrick *et al*., 2014
Redlich-Peterson	$q = \frac{K_{RP} C_e}{1 + \alpha {C_e}^{\beta}}$	$\ln\left[K_{RP}\frac{C_e}{q} - 1\right] = \beta \ln(C_e) + \ln(\alpha)$ $y = \ln\left[K_{RP}\frac{C_e}{q} - 1\right] \quad x = \ln\,(C_e)$	Bhatt *et al*., 2012 and Atulegwu-Patrick *et al*., 2014

Where q and Ce remains as defined above, q_{max} is the maximum amount of metal ion that can be sorbed, K_L is the Langmuir adsorption constant, K_F is the Freundlich constant, n_F is the heterogeneity factor of adsorption sites (dimensionless), K_{RP} is the Redlich-Peterson constant

that is varied to maximize the linear correlation coefficient (R^2), α is constant and β is the Redlich–Peterson exponent (dimensionless) (Atulegwu-Patrick *et al*., 2014 and Bhatt *et al*., 2012). The Langmuir and Freundlich equations are two parameters models while the Redlich-Peterson equation is a three-parameter model that combines the features of both the Langmuir and Freundlich isotherms. A dimensionless constant separation factor R_L further expresses the essential characteristics of Langmuir using equation 3 below:

$$R_{\mathrm{L}} = \frac{1}{1 + K_L C_o} \tag{3}$$

Where C_o and K_L are as described earlier. Adsorption is favourable if the value of $R_L < 1$, linear if = 1, unfavourable if > 1 and irreversible if = 0 (Atulegwu-Patrick *et al*., 2014 and Krishna, 2014).

3.3 Heavy metals analysis by inductively coupled plasma-atomic emission spectroscopy (ICP)

An inductively coupled plasma-atomic emission spectroscopy (SPECTRO CIROS ^{CCD}) was used to analyze and quantify the concentrations of heavy metals present in their respective solutions after centrifugation to remove flocs formed γ-PGA-metal precipitation. All atomic absorption standards (1000 mg/l) were obtained from Sigma-Aldrich, UK and used to prepare working calibration standards (0.1 mg/l – 100 mg/l).

3.4 Statistical analyses

All results were statistically analysed using Microsoft Excel 2010, GraphPad Prism 6 and ORIGINLAB 6. Two-way ANOVA using the Bonferroni multiple comparison test was used for data analysis to determine the difference between individual groups in a data set. A comparison is statistically significant if the P value is ≤ 0.05. Two-way ANOVA was used to compare the variance between the growths of studied bacterial strains at two different temperatures in three culture media at various time intervals.

4.0 TOXIGENIC ANALYSIS OF γ-PGA PRODUCING STRAINS

4.1 Toxigenic analysis

It is important to toxigenically analyse the *Bacillus* strains used in the production of γ-PGA before the application of γ-PGA to ensure that even though these strains are known to be non-pathogenic, they do no not produce toxins under production conditions. Bhat *et al*. (2013) analysed the toxigenicity of *Bacillus subtilis* (natto) ATCC 15245 and found that it was toxin free. Hence, the presence of major enterotoxins and virulence factors in *B. licheniformis* ATCC 9945a and *B. licheniformis* ATCC 9945 were studied and compared to *Bacillus cereus* as a positive control. *Bacillus cereus*, a member of the genus *Bacillus* is a Gram-positive opportunistic pathogen that has been linked to infections. The pathogenicity of *Bacillus cereus* is initiated as a result of its synthesis of several virulence factors such as phospholipases, haemolysins, diarrhoeagenic enterotoxins and emetic toxin (Drobniewski, 1993). The PCR products of *B. licheniformis* ATCC 9945a, *B. licheniformis* ATCC 9945 and *B. cereus* for screening of genes coding for hbl D/A, nheB, sph, piplc, bceT and entFM are presented in **Fig. 4.1**.

The presence of the housekeeping gene pfkA (**Fig. 4.1**) in PCR products of *B. licheniformis* ATCC 9945a (**G**A), *B. licheniformis* ATCC 9945 (**G**L) and *B. cereus* (**G**C) suggests that DNA extraction and amplification were successful. Four out of the six toxins investigated – nhe (**B**C), entFM (**D**C), sph (**E**C) and piplc (**F**C) were found to be present in *B. cereus* while none of these four genes were present in either *B. licheniformis* ATCC 9945a and *B. licheniformis* ATCC 9945. However, the possibility of having genes that are not conserved between bacterial strains of the same species had been reported (Okstad *et al*., 1999). According to their study, even though *Bacillus cereus* belongs to the same subgroup of *Bacillus* species as *Bacillus subtilis* by both phenotypic and rRNA sequence classification, gene organization is not conserved between *B. cereus* ATCC 10987 and *B. subtilis* 168, numerous putative genes are more linked to genes from other bacteria than to *B. subtilis* (or

may not be present in *B. subtilis* 168) and *B. cereus* contains a 155 bp repetitive sequence that is not present in *B. subtilis* (Okstad *et al*., 1999). Evolutionary and bioinformatics methods will need to be combined in the future to determine the presence and/or location of these genes in the investigated strains.

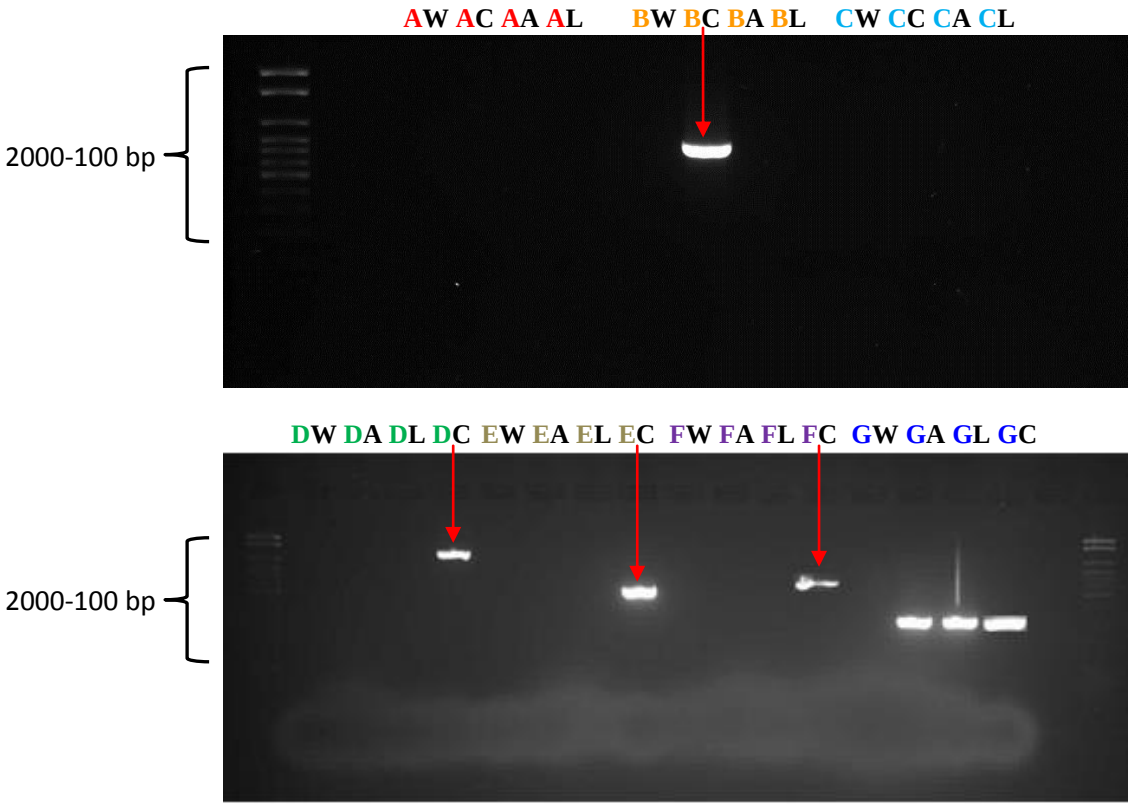


Figure 4.1: PCR products of *B. licheniformis* ATCC 9945a, *B. licheniformis* ATCC 9945 and *B. cereus* for screening of genes coding for toxins. BC, DC, EC and FC indicating the presence of nheB, sph, piplc and entFM in *B. cereus* but not in *B. licheniformis* ATCC 9945a, *B. licheniformis* ATCC 9945

A-hbl-D/A **B**-nheB **C**-bceT **D**-entFM **E**-sph **F**-piplc **G**-pfkA **W**-Water
A- *B. licheniformis* ATCC 9945a **L**- *B. licheniformis* ATCC 9945 **C** - *B. cereus*

Physiological studies were also performed on *B. licheniformis* ATCC 9945a and *B. licheniformis* ATCC 9945 to evaluate their haemolytic and lecithinase potential while using *B. cereus* as a positive control. **Fig. 4.2** shows the test results for haemolytic and lecithinase activities of *B. licheniformis* ATCC 9945a, *B. licheniformis* ATCC 9945 and *B. cereus*.

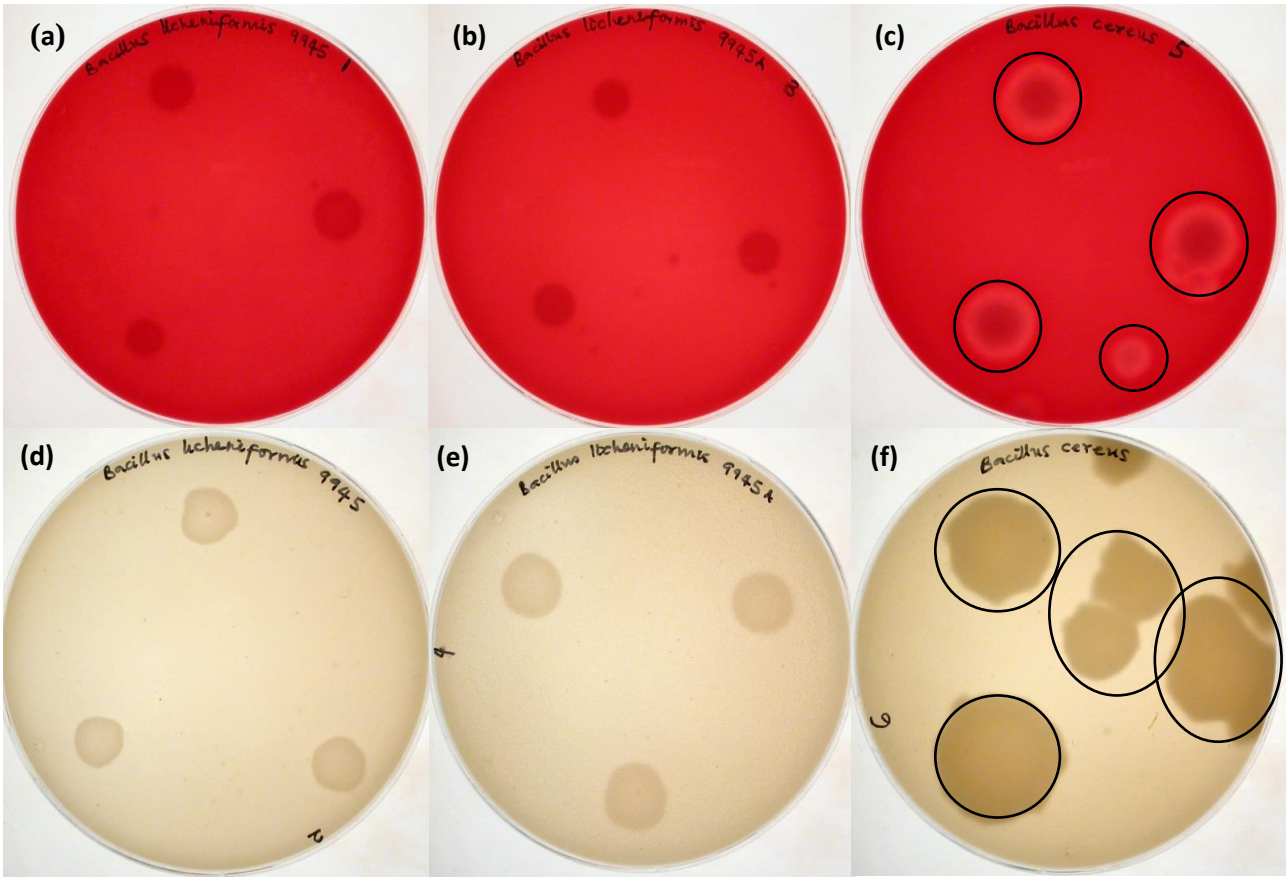


Figure 4.2: Haemolytic and lecithinase activities of *B. licheniformis* ATCC 9945a, *B. licheniformis* ATCC 9945 and *B. cereus*. Haemolytic activities - (a) & (b) no halo seen around the cells indicating absence of haemolytic activity (c) *B. cereus* haemolytic activity showing halo around the cells.
Lecithinase activities - (d) & (e) no halo observed around the cells indicating the absence of lecithinase activity (f) halo around the cells indicating the presence of lecithinase activity.

Bacillus cereus showed haemolytic and lecithinase activities while *B. licheniformis* ATCC 9945 and *B. licheniformis* ATCC 9945a showed neither haemolytic nor lecithinase activity (**Fig. 4.2**). None of the *Bacillus licheniformis* strains used in this study haemolized the blood throughout the 48h of incubation (**Figs. 4.2a & b)** whereas *Bacillus cereus* produced large and clear halos after 48h of growth on 5% sheep blood agar plates at 30°C as highlighted in circles (**Fig. 4.2c**). *Bacillus cereus* also exhibited lecithinase activity with production of opaque halos around colonies after 48h of growth on 8% egg yolk emulsion supplemented nutrient agar plate (**Fig. 4.2f**) while no halo formation was seen around colonies of *B. licheniformis* ATCC 9945 and *B. licheniformis* ATCC 9945a (**Figs. 4.2d & e**).

5.0 RESULTS - BACTERIAL PRODUCTION OF POLY-GAMMA-GLUTAMIC ACID (γ-PGA)

5.1 Introduction

This research investigated the production of γ-PGA using five different *Bacillus* strains in GS medium, medium C and medium E. The strains include *Bacillus licheniformis* 1525, *Bacillus licheniformis* NCTC 6816, *Bacillus licheniformis* ATCC 9945a, *Bacillus licheniformis* ATCC 9945 (also known as *Bacillus licheniformis* CCRC 12826) and *Bacillus subtilis* (natto) ATCC 15245 (also known as *Bacillus natto* Sawamura). During the course of production, aliquots were taken at intervals to assess bacterial growth using plate count method. Some *Bacillus* species such as *Bacillus coagulans* and *Bacillus licheniformis* have been found to be thermophilic bacteria, growing at high temperature up to 70^{o}C and can produce xylanase at an optimum growth temperature of 50^{o}C within 60 hours (Abou-Dobara *et al*., 2011). Thus, this led to the investigation of the effects of temperature (37^{o}C and 50^{o}C) with time on growth and γ-PGA production by *Bacillus licheniformis* 1525, *Bacillus licheniformis* NCTC 6816, *Bacillus licheniformis* ATCC 9945a, *Bacillus licheniformis* ATCC 9945 and *Bacillus subtilis* (natto) ATCC 15245. This research further investigated the effect of culture media on the growth and production of γ-PGA by the strains studied. The effects of NaCl concentration and a precursor – citric acid on the molecular weight of γ-PGA produced by *Bacillus subtilis* (natto) ATCC 15245 and *Bacillus licheniformis* ATCC 9945a in GS medium and medium C respectively were also assessed. FT-IR and NMR were used to identify and determine the structure of the biopolymer respectively. The amount of γ-PGA produced by each bacterium in the different growth media was also evaluated after the fermentation process to determine which medium is best for the growth and production of γ-PGA by each bacterium in terms of yield. The yield of γ-PGA produced differs for each bacterium in each medium. Hence, the characterization of γ-PGA as regards its form (free acid or salt), crystallinity and molecular weight is very important for a good quality product as well as its application. ICP-AES was used to assess the form (free acid or salt) of γ-PGA produced. The crystallinity of the

polymer was evaluated using XRD while aqueous based GPC was used to determine the molecular weight of the polymer. It was discovered that like the yield, the form, crystallinity and molecular weight of γ-PGA differs for each bacterium in each medium.

5.2 Growth assessment of *Bacillus* strains

Investigated strains - *Bacillus licheniformis* 1525, *Bacillus licheniformis* NCTC 6816, *Bacillus licheniformis* ATCC 9945a, *Bacillus licheniformis* ATCC 9945 and *Bacillus subtilis* (natto) ATCC 15245 were grown in GS, C and E media both at 37^{o}C and 50^{o}C and at 150 rpm for 96 hours in shake flasks with 5% inoculum level and the pH of the medium adjusted to 6.8 - 7.2 to study the production of γ-PGA. Aliquots were taken at time zero (0 hour) and at intervals (4, 24, 48, 72 and 96 hours) to determine the pH of each medium and bacterial growth. The investigation of the growth of different bacteria in the different media was essential for the determination of the appropriate medium for each bacterium for the production of γ-PGA as the composition of one medium varies from the other. The Miles and Misra plating method was used to examine the growth of all used bacterial strains in GS, C and E media. Colonies of cells that appeared on plates after overnight incubation at 30^{o}C were counted, common logarithm ($\log_{10}$) of the number of colony forming units (CFU/ml) were derived and used to construct growth curves (**Figs. 5.1 – 5.5**). All experiments were carried out in triplicates and analysed statistically.

5.2.1 *Bacillus licheniformis* 1525

For bacterial growth of *Bacillus licheniformis* 1525 in GS medium at 37^{o}C, a gradual increase in bacterial growth was observed at 0 hour to 4 hours of incubation. The mean cell population however increased from $\log_{10}$ 8.2 at 4 hours of incubation to 8.96 after 24 hour of incubation. The maximum mean cell population of $\log_{10}$ 9.52 was obtained at 48 hours. Cell growth became stationary between 48 and 72 hours, and then began to decline after 72 hours of incubation (**Fig. 5.1a**). At 50^{o}C, maximum mean cell population of $\log_{10}$ 8.54 was attained at 24 hours of growth followed by a rapid decline in cell population to $\log_{10}$ 7.54 at 96 hours (**Fig. 5.1b**).

In medium C, at 37^{o}C, a similar growth pattern to that of GS medium was observed. A maximum cell counts of $\log_{10}$ 9.45 was attained after 48 hours which was followed by a stationary phase between 48 and 72 hours before reduction in cell population (**Fig. 5.1a**). Bacterial cell count reached a maximum of $\log_{10}$ 8.55 by 24 hours when *Bacillus licheniformis* 1525 was grown at 50^{o}C, after which cells began to die (**Fig. 5.1b**).

Cell count increased from $\log_{10}$ 8.15 at 0 hour to $\log_{10}$ 8.65 by 24 hours when *Bacillus licheniformis* 1525 was cultured at 37^{o}C in medium E. Unlike in GS and C media, maximum cell population of $\log_{10}$ 9.73 was observed after 72 hours of growth before a short stationary phase (**Fig. 5.1a**). While at 50^{o}C, highest mean cell count of $\log_{10}$ 8.54 was recorded at 24 hours of cultivation followed by rapid reduction in cell population to $\log_{10}$ 7.96 by the end of fermentation (**Fig. 5.1b**).

Statistically, the differences between the growth of *Bacillus licheniformis* 1525 at 37^{o}C and 50^{o}C in all media were extremely significant ($P < 0.0001$) (**Appendix 11.3**). There were no significant differences between the growths of *Bacillus licheniformis* 1525 in GS medium and medium C at 37^{o}C while high significant differences ($P < 0.0001$) were observed between

the growths of *Bacillus licheniformis* 1525 in GS medium vs medium E and Medium C vs medium E at 24, 72 and 96 hours (**Appendix 11.1**). At 50°C, there were no significant differences between the growths of *Bacillus licheniformis* 1525 in all media before 72 hours, a significant difference between GS medium and medium C (P = 0.0454 at 96 hours, a high significant differences between GS medium and medium E (P < 0.0001) and between medium C and medium E (P< 0.0006) at 72 and 96 hours (**Appendix 11.2**).

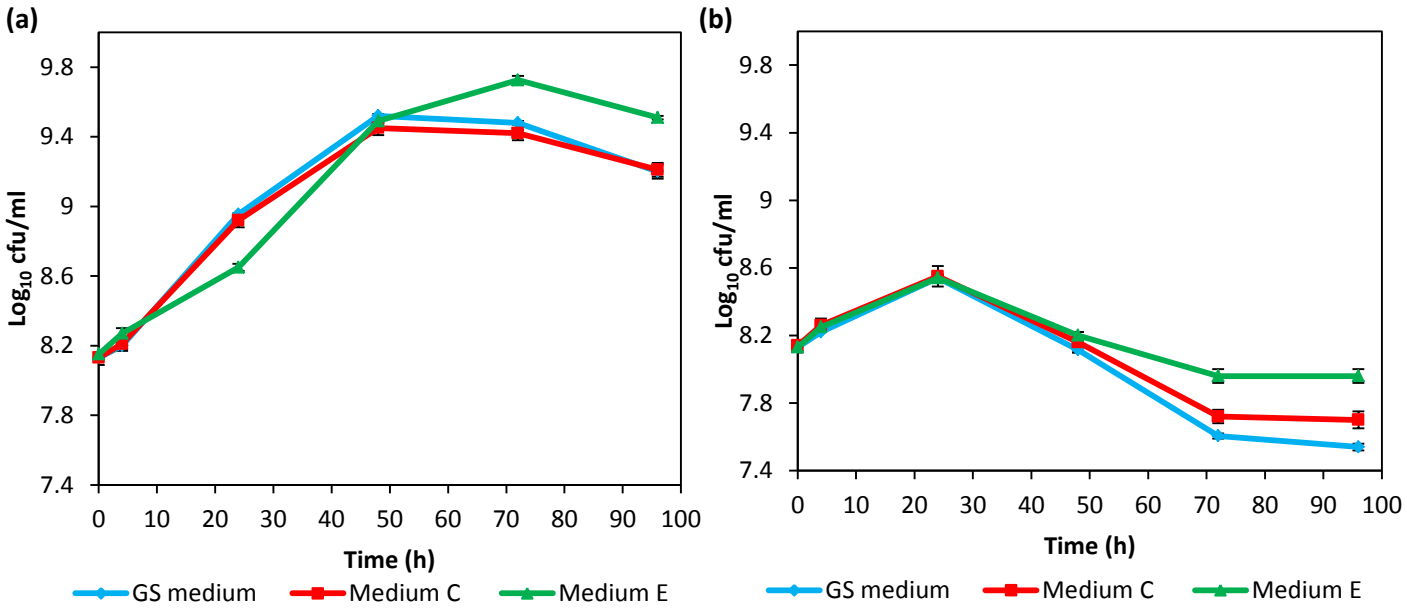


Figure 5.1: Growth curves (mean ± SEM) for *B. licheniformis* 1525 when grown in GS medium, medium C and medium E at 150 rpm and (a) 37°C and (b) 50°C for 96 hours to evaluate the production of γ-PGA (n=3)

5.2.2 *Bacillus licheniformis* NCTC 6816

In GS medium and at 37°C, a gradual increase in the growth of *B. licheniformis* NCTC 6816 was observed between 0 and 4 hours of incubation followed by a rapid increase thereafter until maximum cell population of $\log_{10}$ 9.05 was reached at 48 hours (**Fig. 5.2a**), after which cell population started declining. On the other hand, at 50°C, maximum mean cell count of $\log_{10}$ 8.46 (**Fig. 5.2b**) was recorded after 24 hours of growth followed by drastic decline in cell population to $\log_{10}$ 7.22 at 96 hours.

Like GS medium, a steady growth was observed between 0 and 4 hours of incubation when cells were grown in medium C at 37^{o}C. The mean cell count however increased from $\log_{10}$ 7.95 at 4 hours of incubation to $\log_{10}$ 8.9 after 24 hour of incubation. The maximum mean cell population of $\log_{10}$ 9.07 was obtained at 48 hours (**Fig. 5.2a**). Cell growth then began to decline gradually thereafter. At 50^{o}C, cell population gradually increased from $\log_{10}$ 7.75 at 0 hour to $\log_{10}$ 8.43 which was the maximum cell population at 24 hours (**Fig. 5.2b**). This was then followed by a drastic loss in viability to $\log_{10}$ 7.19 at 96 hours.

In medium E, growth was slightly different. At 37^{o}C, a steady growth pattern was observed with a maximum cell count of $\log_{10}$ 9.15 recorded after 72 hours of cultivation before cell population only slightly declined to $\log_{10}$ 9.00 after 96 hours (**Fig. 5.2a**). It was observed that even though there was a slight reduction in cell population from 72 hours to 96 hours, the amount of cells viable at 96 hours in medium E was still much higher than for GS and C media. Whereas, at 50^{o}C, maximum cell population of $\log_{10}$ 8.54 was achieved after 48 hours of growth just before cell viability started to decline drastically in the culture (**Fig. 5.2b**).

Statistically, there were extreme significant differences between the growth of *B. licheniformis* NCTC 6816 at 37^{o}C and 50^{o}C in all media ($P < 0.0001$). There were no significant differences between the growths of *B. licheniformis* NCTC 6816 in GS medium and medium C at 37^{o}C ($P > 0.999$) but great significant differences in GS medium vs medium E ($P < 0.0001$) and Medium C vs medium E ($P < 0.0001$) at 24, 72 and 96 hours (**Appendix 11.4**). Whereas, at 50^{o}C, there were no significant differences in the growths of *B. licheniformis* NCTC 6816 in GS, C and E media (**Appendix 11.5)**

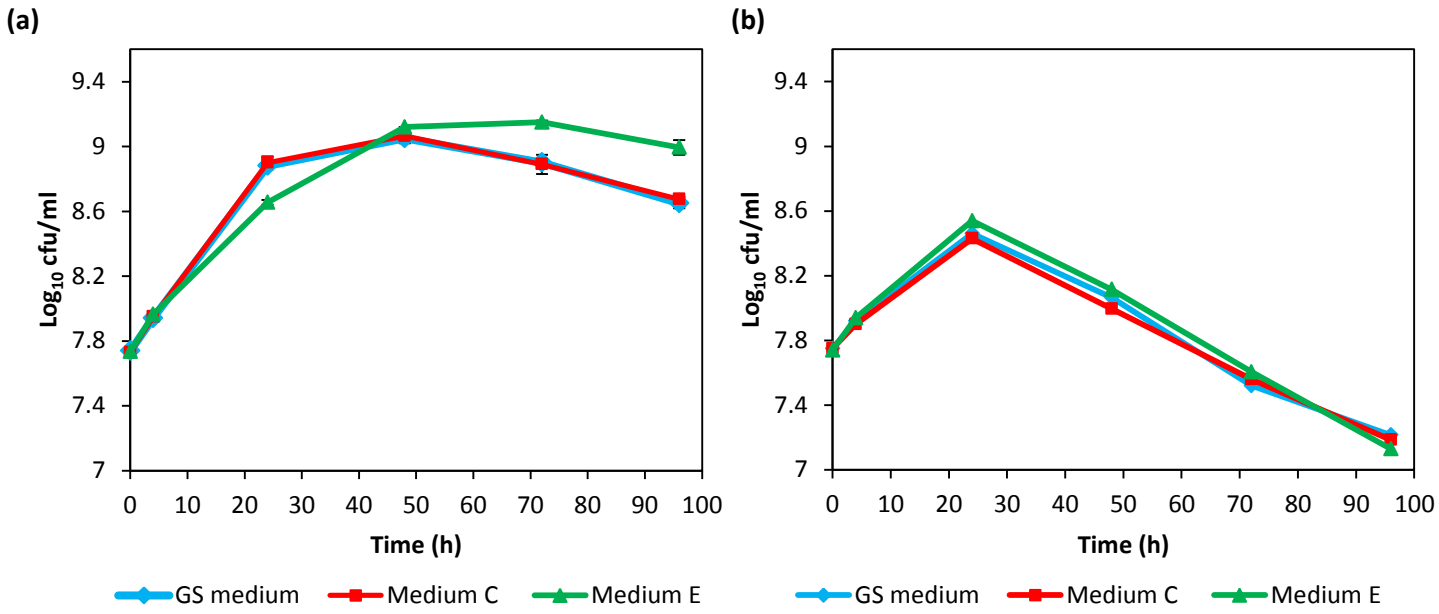


Figure 5.2: Growth curves (mean ± SEM) for *B. licheniformis* NCTC 6816 when grown in GS medium, medium C and medium E at 150 rpm and (a) 37°C and (b) 50°C for 96 hours to evaluate the production of γ-PGA (n=3)

5.2.3 *Bacillus licheniformis* ATCC 9945a

For the growth of *Bacillus licheniformis* ATCC 9945a in GS medium at 37°C, a rapid increase in cell population was observed in the first 24 hours of growth with a maximum cell count of $\log_{10}$ 8.77 (**Fig 5.3a**). This was followed by a stationary phase between 24 hours and 48 hours of cultivation before cells started losing viability. When cultivated at 50°C, the cells grew slowly from $\log_{10}$ 7.28 at time 0 to $\log_{10}$ 7.65 after 24 hours. This was followed by a rapid decline to $\log_{10}$ 6.77 due to huge loss in cell viability (**Fig 5.3b**).

When *Bacillus licheniformis* ATCC 9945a was grown at 37°C in medium C, a similar growth pattern to that of GS medium was observed. Mean cell count increased rapidly from $\log_{10}$ 7.29 at 0 hour of incubation to $\log_{10}$ 8.74 after 24 hours of incubation. Maximum cell population of $\log_{10}$ 8.75 was reached after 48 hours of growth just before cell population started declining (**Fig 5.3a**). While at 50°C, cell population increased slowly from $\log_{10}$ 7.32 at time 0 to a maximum of $\log_{10}$ 7.65 after 24 hours (**Fig 5.3b**). Thereafter, cell population in the culture broth decreased radically to $\log_{10}$ 6.69 at 96 hours.

Cell count increased from $\log_{10}$ 7.29 at 0 hour to $\log_{10}$ 8.71 by 24 hours when *Bacillus licheniformis* ATCC 9945a was grown in medium E at 37°C (**Fig 5.3a**). Maximum mean cell population of $\log_{10}$ 8.79 was observed after 48 hours of growth before cells began to decline. Whereas at 50°C, highest mean cell count of $\log_{10}$ 7.64 was recorded after 24 hours of cultivation followed by a rapid decrease in cell population to $\log_{10}$ 6.62 after 96 hours of incubation (**Fig 5.3b**).

The growth of *B. licheniformis* ATCC 9945a at 37°C was highly significantly different ($P < 0.0001$) from the growths of *B. licheniformis* ATCC 9945a at 50°C in all media (**Appendix 11.9**). There were no significant differences ($P > 0.999$) between the growth of *B. licheniformis* ATCC 9945a in GS medium vs medium C, GS medium vs medium E and Medium C vs medium E from 0 – 48 hours (**Appendix 11.7**), but a very significant difference between GS medium and medium E at 72 hours ($P = 0.0025$) and 96 hours ($P = 0.0003$). At 50°C, there were no significant differences ($P > 0.9999$) in the growth of *B. licheniformis* ATCC 9945a in GS, C and E media (**Appendix 11.8**).

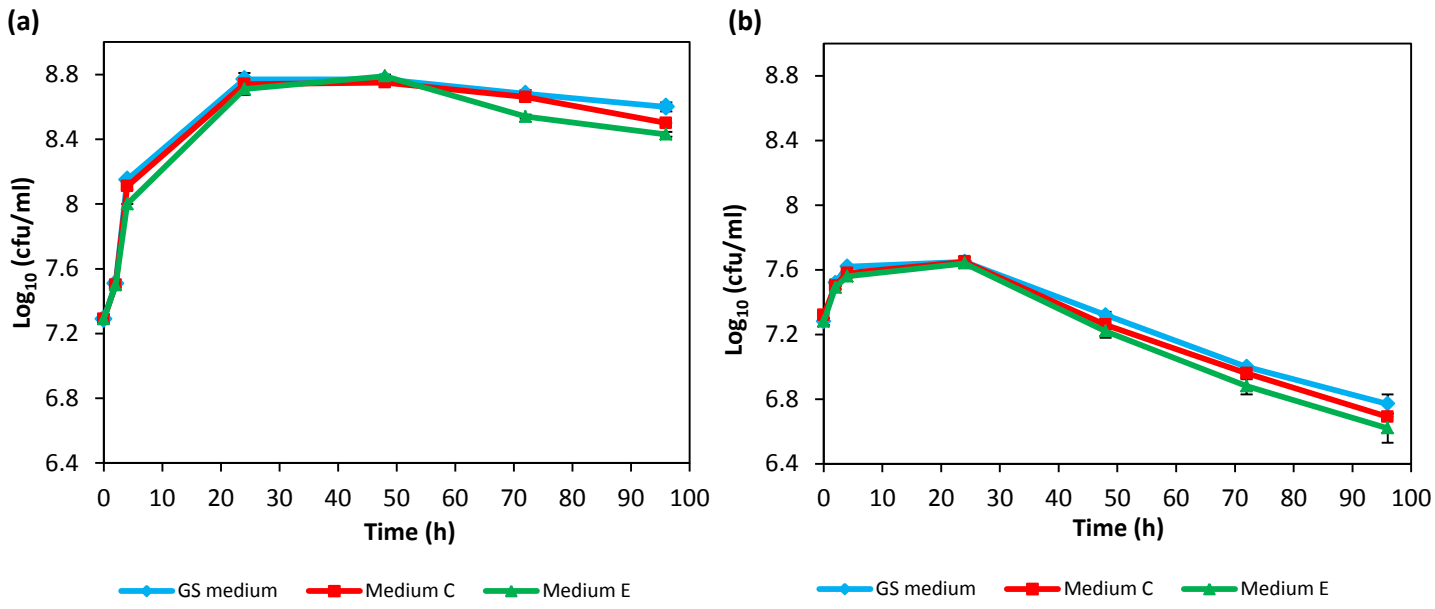


Figure 5.3: Growth curves (mean ± SEM) for *B. licheniformis* ATCC 9945a when grown in GS medium, medium C and medium E at 150 rpm and (a) 37°C and (b) 50°C for 96 hours to evaluate the production of γ-PGA (n=3)

5.2.4 *Bacillus licheniformis* ATCC 9945

In GS medium, the cell population of *Bacillus licheniformis* ATCC 9945 increased gradually from $\log_{10}$ 7.58 at time 0 to $\log_{10}$ 8.61 after 24 hours when grown at 37^{o}C. Maximum cell count of $\log_{10}$ 8.69 was achieved after 48 hours of cultivation before cell population in the culture started to decline (**Fig 5.4a**). On the other hand, when *Bacillus licheniformis* ATCC 9945 was cultivated in GS medium at 50^{o}C, maximum mean cell count of $\log_{10}$ 7.68 was recorded after 24 hours followed by radical loss in cell viability to $\log_{10}$ 6.78 at 96 hours (**Fig 5.4b**).

When *Bacillus licheniformis* ATCC 9945 was grown at 37^{o}C in medium C, cell population increased from $\log_{10}$ 7.56 to a maximum cell count of $\log_{10}$ 8.62 after 48 hours of growth (**Fig 5.4a**). After 48 hours, cell population in the culture started to decrease. At 50^{o}C, cell population increased slowly from $\log_{10}$ 7.22 to $\log_{10}$ 7.68 at 24 hours before rapid reduction in cell population commenced (**Fig 5.4b**).

For the growth of *Bacillus licheniformis* ATCC 9945 at 37^{o}C in medium E, a rapid increase in cell population was observed in the first 24 hours of growth (increasing from $\log_{10}$ 7.56 to $\log_{10}$ 8.66). Growth continued gradually until after 72 hours with maximum cell count of $\log_{10}$ 8.76 (**Fig 5.4a**) before the cell population only slightly decreased to $\log_{10}$ 8.63 in the culture medium. It was also observed that even though there was a loss in viability of cells at the end of fermentation, the amount of cells viable at 96 hours in medium E was still much higher than for GS and C media. When cultivated at 50^{o}C, the cells grew slowly from $\log_{10}$ 7.26 at time 0 to $\log_{10}$ 7.70 after 24 hours (**Fig 5.4b**). This was followed by a rapid decline due to great loss in cell viability to $\log_{10}$ 6.76.

Following statistical analysis, the differences between the growths of *Bacillus licheniformis* ATCC 9945 at 37^{o}C and 50^{o}C in all media were extremely significant ($P < 0.0001$)

(**Appendix 11.12**). There were no significant differences (P > 0.09) between the growth of *B. licheniformis* ATCC 9945 in GS medium vs medium C, GS medium vs medium E and Medium C vs medium E from 0 – 48, but a very significant difference between GS medium and medium C (P = 0.0072) at 72 hours, a significant difference between GS medium and medium C (P = 0.0202) at 96 hours and extremely significant differences in GS medium vs medium E and medium C vs medium E (P < 0.0001) at 72 and 96 hours at 37^{o}C (**Appendix 11.10**). At 50^{o}C, there were no significant differences (P > 0.9999) in the growth of *B. licheniformis* ATCC 9945 in GS, C and E media at 24 and 96 hours (**Appendix 11.11)**

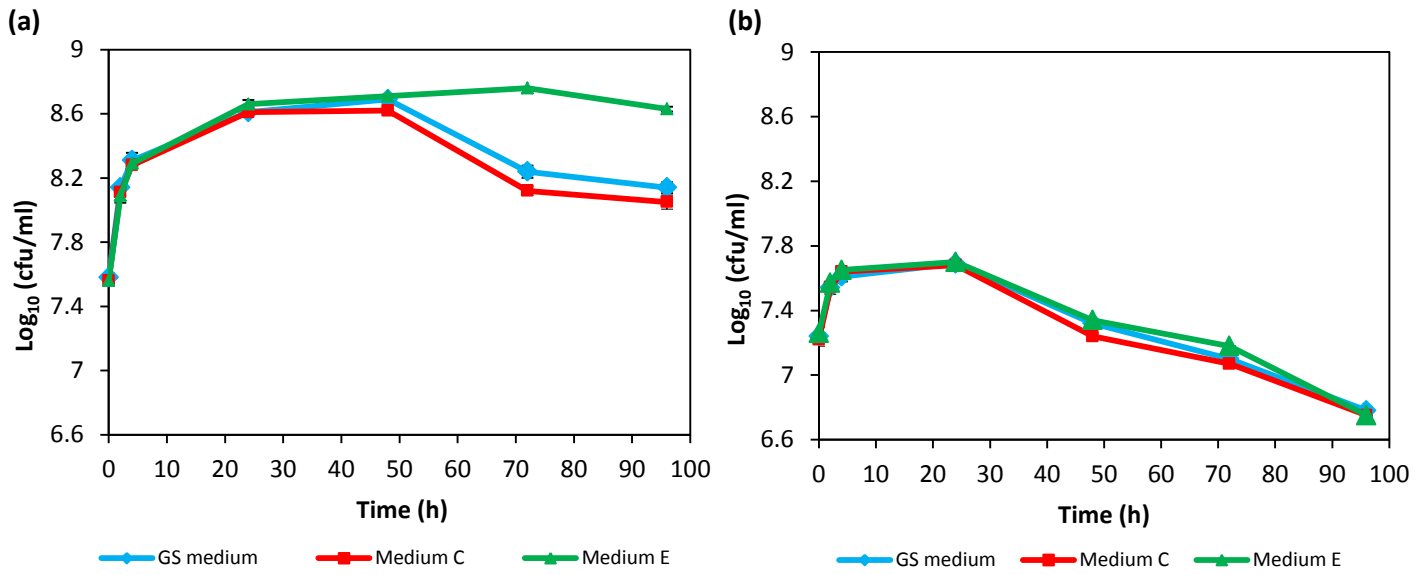


Figure 5.4: Growth curves (mean ± SEM) for *B. licheniformis* ATCC 9945 when grown in GS medium, medium C and medium E at 150 rpm and (a) 37^{o}C and (b) 50^{o}C for 96 hours to evaluate the production of γ-PGA (n=3)

5.2.5 *Bacillus subtilis* (natto) ATCC 15245

When *B. subtilis* (natto) ATCC 15245 was grown in GS medium at 37^{o}C, a rapid increase in growth was observed between 0 and 24 hours of incubation. The maximum mean cell population of $\log_{10}$ 8.74 was attained after 24 hours of incubation. Cell population however dropped thereafter to $\log_{10}$ 8.32 at 48 hours and was followed by stationary phase between 48 hours and 72 hours before cell population slightly increased again to $\log_{10}$ 8.43 after 96 hours

of growth (**Fig. 5.5a**). When grown at 50^{o}C, cell population increased slowly from $\log_{10}$ 7.19 at 0 hour to a maximum of $\log_{10}$ 7.48 after 24 hours (**Fig. 5.5b**), after which cell population in the culture broth decreased to $\log_{10}$ 6.78 after 96 hours of cultivation due to cell death.

When *B. subtilis* (natto) ATCC 15245 was cultivated at 37^{o}C in medium C, a rapid increase in cell population was observed between 0 and 24 hours of growth just like it was seen in GS medium. Maximum cell population of $\log_{10}$ 8.69 was reached after 24 hours of growth before a short phase of declination and then stationary phase between 48 and 72 hours (**Fig. 5.5a**). Cells then became viable again after 72 hours. While at 50^{o}C, highest mean cell count of $\log_{10}$ 7.50 was attained at 24 hours just before a rapid decline in the population of cells to $\log_{10}$ 6.78 after 96 hours (**Fig. 5.5b**).

For the growth of *B. subtilis* (natto) ATCC 15245 in medium E, at 37^{o}C, a maximum cell population of $\log_{10}$ 8.64 was attained after 48 hours of incubation unlike in GS and C medium. This was followed by a slight decrease in the cell population (**Fig. 5.5a**). At 50^{o}C, in a similar pattern to what was observed in GS and C media, maximum mean cell population of $\log_{10}$ 7.42 was reached after 24 hours (**Fig. 5.5b**), followed by a rapid decrease in cell population to $\log_{10}$ 6.64 after 96 hours.

The differences between the growth of *B. subtilis (natto)* ATCC 15245 at 37^{o}C and 50^{o}C in all media were extremely significant ($P < 0.0001$) (**Appendix 11.15**). There were no significant differences in the growth of *B. subtilis (natto)* ATCC 15245 at 37^{o}C between GS medium, medium C and Medium E before 48 hours ($P \geq 0.3998$), but highly significant differences ($P < 0.0001$) between GS medium vs medium E and medium C vs medium E at 48 hours of growth, significant differences between GS medium and medium E ($P = 0.0396$) and between medium C and medium E ($P = 0.0157$) at 72 hours (**Appendix 11.13**). At 50^{o}C, there were no significant differences in the growths of *B. subtilis (natto)* ATCC 15245 in all

media at 24 hours. However, the differences between the growths of *B. subtilis (natto)* ATCC 15245 in GS medium vs medium E and medium C vs medium E were extremely significant ($P < 0.0001$) at 48 hours and significant ($P = 0.0111$) at 96 hours (**Appendix 11.14**).

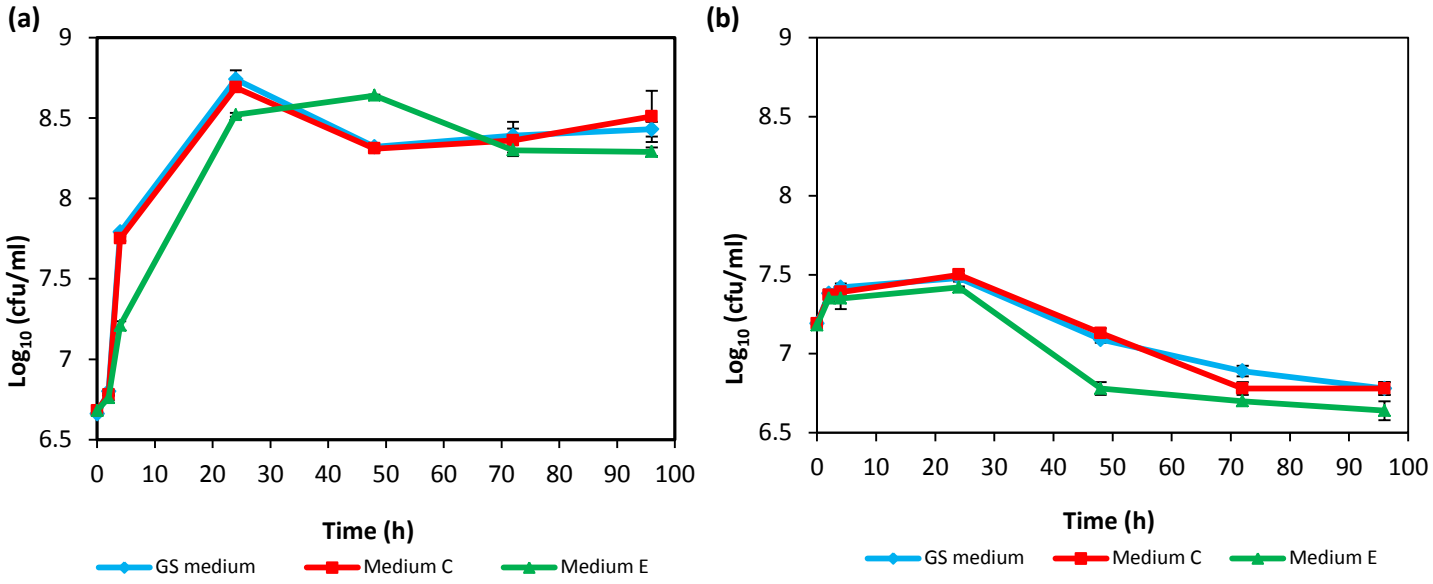


Figure 5.5: Growth curves (mean ± SEM) for *B. subtilis* (natto) ATCC 15245 when grown in GS medium, medium C and medium E at 150 rpm and (a) 37°C and (b) 50°C for 96 hours to evaluate the production of γ-PGA (n=3)

5.3 pH changes with time

For growth and production of γ-PGA in GS, C and E media, aliquots were taken at time zero and after every 24 hours to determine the pH of each medium. Results (**Table 5.1**) showed that for growths and productions in GS and C media, initial pH of 6.8 decreased to ~6.5 after 48 hours until the end of fermentation while the initial pH of 7.2 decreased to about 6.5 at 48 hours and later increased to 6.8 at 96 hours.

Table 5.1: pH changes with time in GS, C and E media

	GS medium (pH)	Medium C (pH)	Medium E (pH)
0 h	6.80	6.80	7.2
24 h	6.63	6.61	6.78
48 h	6.51	6.50	6.52
72 h	6.53	6.50	6.69
96 h	6.53	6.51	6.81

5.4 Summary of *Bacilli* growth for γ-PGA production

The growths of five *Bacillus* strains in GS, C and E media at 37^{o}C and 50^{o}C were investigated. Samples were taken at regular intervals during fermentation to analyse cell growth.

At 50^{o}C, in all media, all investigated strains reached their maximum cell population at 24 hours followed by rapid decline in cell population **(Figs. 5.1b, 5.2b, 5.3b, 5.4b & 5.5b)**. As a result, further cultivation at 50^{o}C was discontinued.

Fig. 5.6 shows the growth of all investigated strains in GS, C and E media at 37^{o}C. In medium E, all *B. licheniformis* strains under study (with the exception of *B. licheniformis* ATCC 9945a) reached their maximum cell count at 72 hours (**Fig. 5.6c**) while in both GS and C media, all *B. licheniformis* strains attained theirs at 48 hours (**Figs. 5.6a & b).** The growth of *B. licheniformis* ATCC 9945a in all media (GS, C & E) reached its maximum at 48 hours (**Fig. 5.6**). The growth of *B. subtilis (natto)* ATCC 15245 in medium E reached its maximum at 48 hours before a decrease in cell population whereas, in medium C and GS, maximum cell count was attained at 24 hours before notable loss in cell viability at 48 hours and then an increase in cell population after 72 hours (**Figs. 5.6a & b**).

Overall, the growth patterns of all strains in GS and C media were similar, probably because of their similar medium composition. For the growth of *Bacillus licheniformis* 1525, *Bacillus licheniformis* NCTC 6816 and *Bacillus licheniformis* ATCC 9945 in medium E, even though the number of viable cells decreased after 72 hours, the amount of cells viable at 96 hours in medium E was still much higher than for GS and C media.

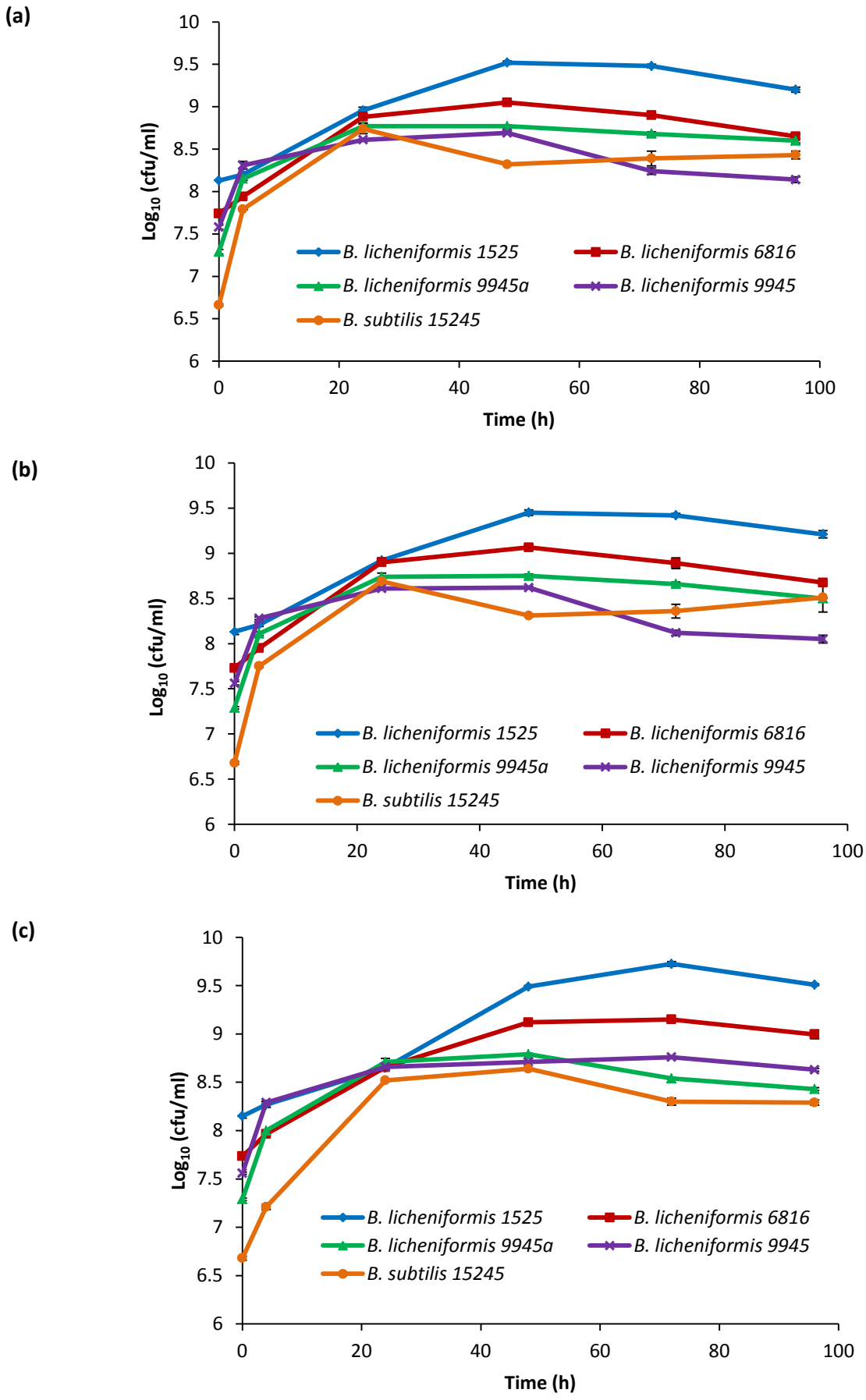


Figure 5.6: Growth curves (mean ± SEM) for all 5 investigated *Bacillus* strains in (a) GS medium, (b) medium C and (c) medium E at 37°C and 150 rpm for 96 hours. Tests were carried out in triplicates (n=3)

5.5 Polymer yield

The understanding of how the yield of γ-PGA differs with bacteria producing them as well medium of production is essential as this would help identify which medium is best for the cultivation of each bacterial strain for γ-PGA production.

Previous studies by Shih *et al*. (2001) and Berekaa *et al*. (2009) had indicated that maximum γ-PGA production during batch fermentation was obtained after 96 hours of incubation. Therefore, to investigate the yield and effect of different culture media on the yield of γ-PGA produced by the bacterial strains used in this study, each strain was grown in GS medium, medium C and medium E and incubated aerobically in a shaker at 37^{o}C for 96 hours. After every 96 hours of cultivation, polymers produced were purified and obtained as dry powders and then weighed to determine the yield. **Fig 5.7** represents the yields of γ-PGA produced by the five bacteria in GS medium, medium C and medium E.

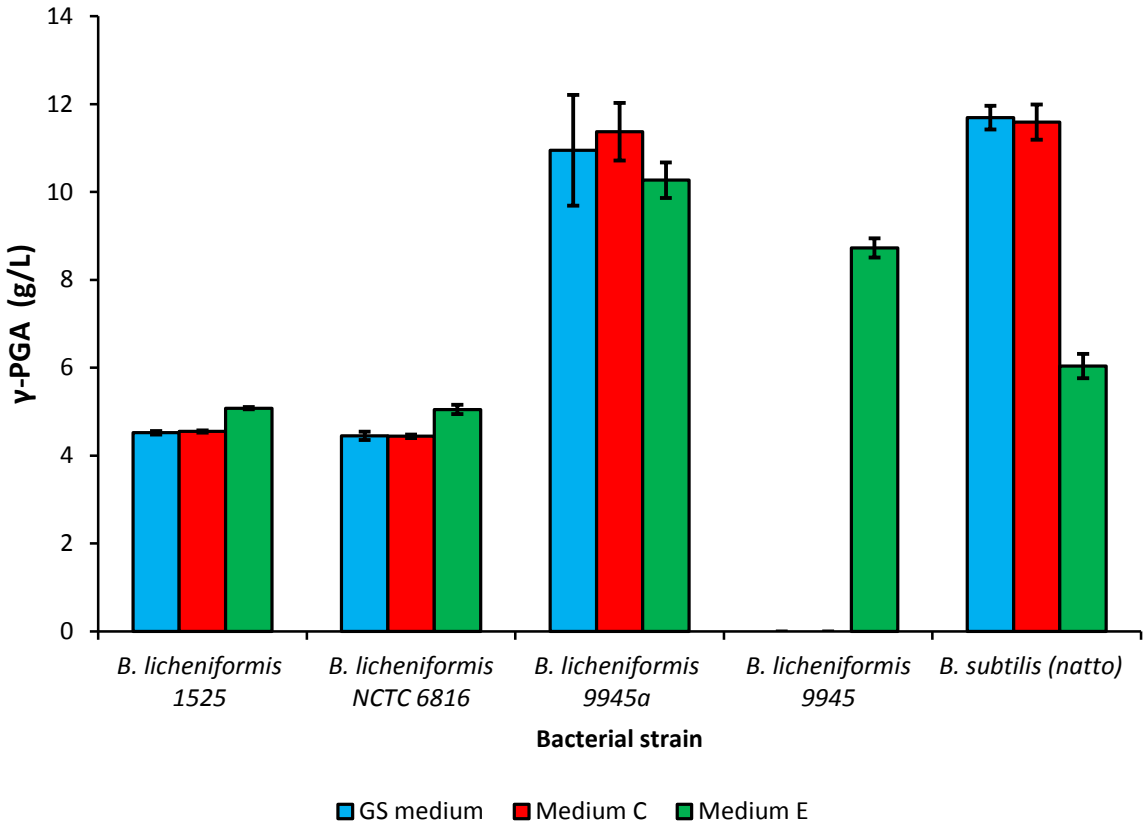


Figure 5.7: Yields (mean ± SEM) of γ-PGA (g/L) produced by different *Bacillus* strains in GS medium, medium C and medium E at 37^{o}C for 96 hours. *Bacillus licheniformis* ATCC 9945 did not give any yield in GS medium and medium C

The yields of γ-PGA produced by *Bacillus licheniformis* 1525 and *Bacillus licheniformis* NCTC 6816 in GS medium, medium C and medium E were similar and not significantly different (P > 0.9999) (**Appendix 11.16a & b**). The yields of γ-PGA produced by *Bacillus licheniformis* ATCC 9945a in GS medium, medium C and medium E were also comparable and not significantly different (P > 0.9999) (**Appendix 11.16a**). However, *B. subtilis* (natto) ATCC 15245 produced significantly higher γ-PGA in GS medium and medium C than in medium E (P < 0.0001) (**Appendix 11.16a**). γ-PGA was not produced by *B. licheniformis* ATCC 9945 in both GS medium and medium C, despite initial growth in both media. This organism did however produce significantly high yield in medium E. The results clearly show *B. licheniformis* ATCC 9945a to produce double the yield of other *B. licheniformis* strains (except *B. licheniformis* ATCC 9945). *B. subtilis* (natto) ATCC 15245 also produced high yields but only in GS and C media.

5.6 Identification of γ-PGA using FT-IR spectroscopy

To identify the isolated polymers, the polymers were analysed using FT-IR and the resultant scans were compared to commercial standard samples of γ-PGA. Each spectrum presented is an average of three absorption spectra. Polymers recovered from GS medium and medium C were off-white crystalline powder while those from medium E were brownish in colour.

Figs. 5.8 to 5.12 show the infra-red (IR) absorption spectra of the different γ-PGA produced by the five strains under investigation as compared to standard samples. The FT-IR absorption spectra of the γ-PGA samples produced in this study were similar and comparable to that of the commercial γ-PGA sample. On comparison with the spectrum of the commercial sample, it was observed that each peak in each spectrum corresponds to a specific bond in the compound.

The FT-IR absorption spectra of γ-PGA samples displayed characteristic strong hydroxyl absorption at about 3350-3450 cm^{-1}, carbonyl absorption at about 1395-1410 and C-N groups absorption peaks from 1070 cm^{-1} to 1138 cm^{-1}. The strong absorption peaks observed at approximately 620 cm^{-1} are characteristic of and N-H oop bending. The absorption peak at about 1738 cm^{-1} show distinctive C=O stretch while the peaks ranging from 1575 cm^{-1} to 1619 cm^{-1} are characteristics of amide groups. The spectra of all γ-PGA samples (**Figs. 5.8 to 5.12**) showed all of these characteristic peaks.

Consequently, the polymers produced by all bacteria at 37^{o}C after 96 hours and in all culture media (with the exception of *Bacillus licheniformis* ATCC 9945 in GS and C media) were identified as γ-PGA.

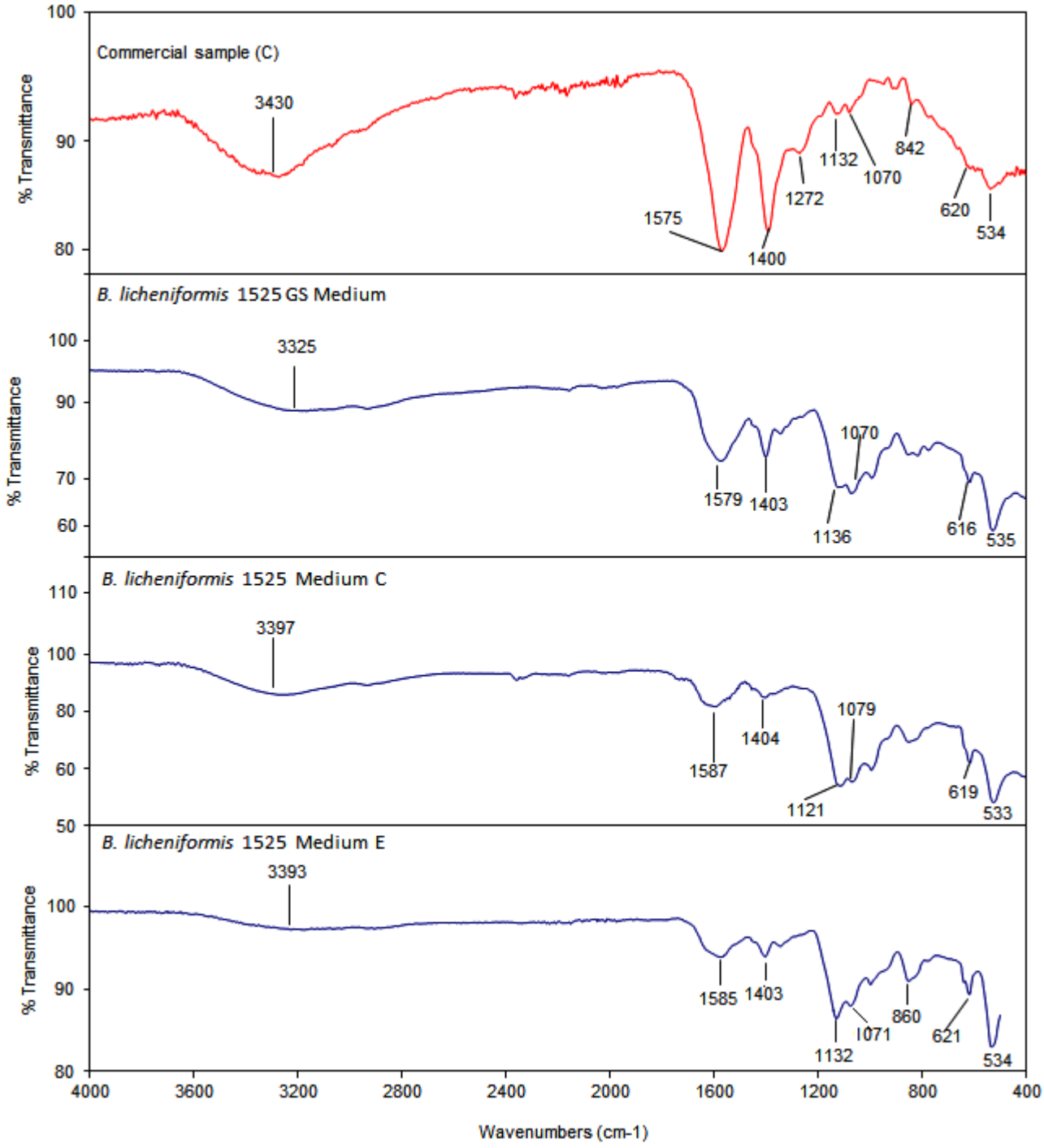


Figure 5.8: FT-IR spectra of γ-PGA(s) produced by *B. licheniformis* 1525 in GS, C and E media at 37ºC and 150 rpm for 96 hours compared to the spectra of a commercial γ-PGA sample. Each spectrum is an average of 3 spectra (n=3)

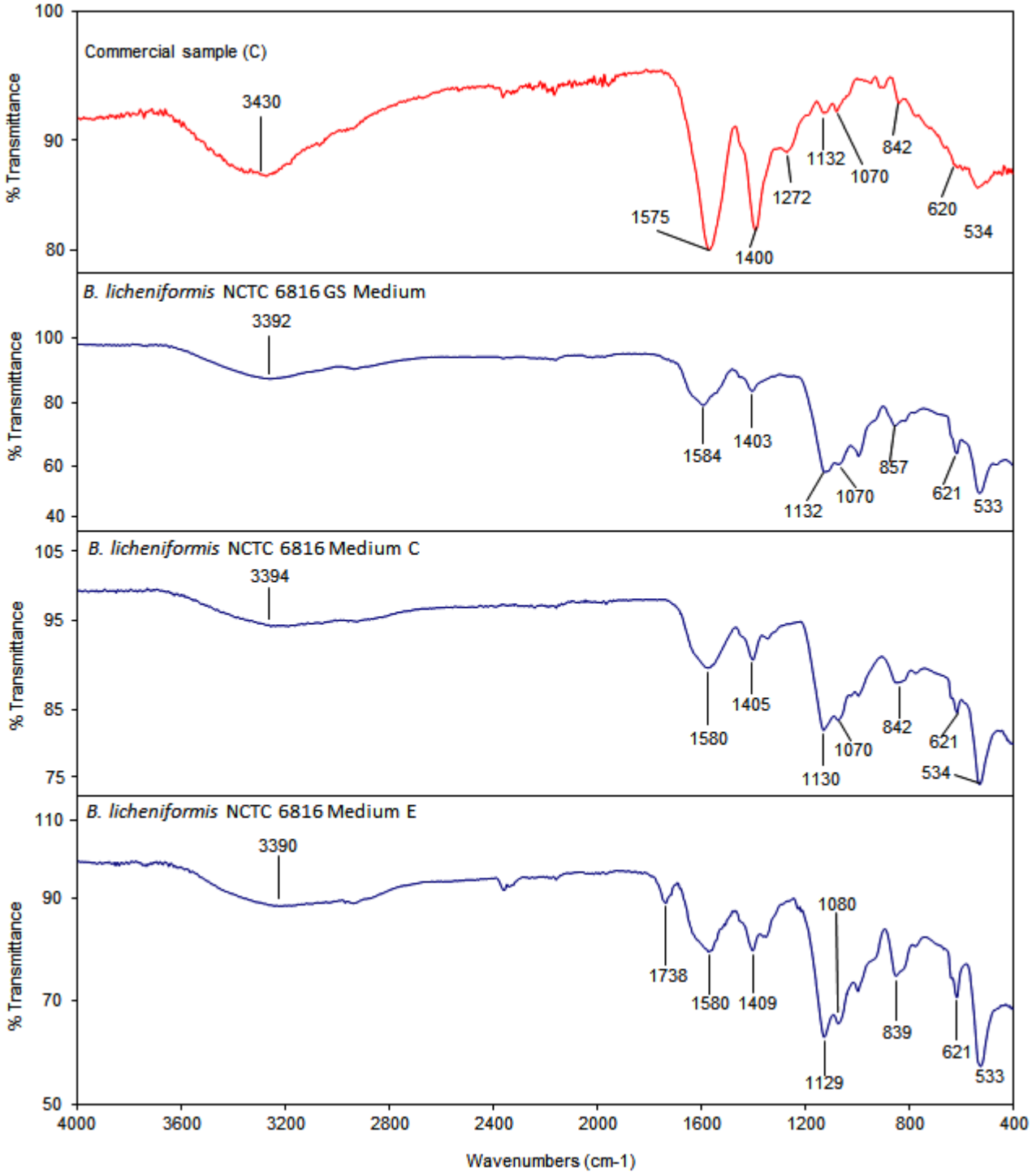


Figure 5.9: FT-IR spectra of γ-PGA(s) produced by *B. licheniformis* NCTC 6816 in GS, C and E media at 37ºC and 150 rpm for 96 hours compared to the spectra of a commercial γ-PGA sample. Each spectrum is an average of 3 spectra (n=3)

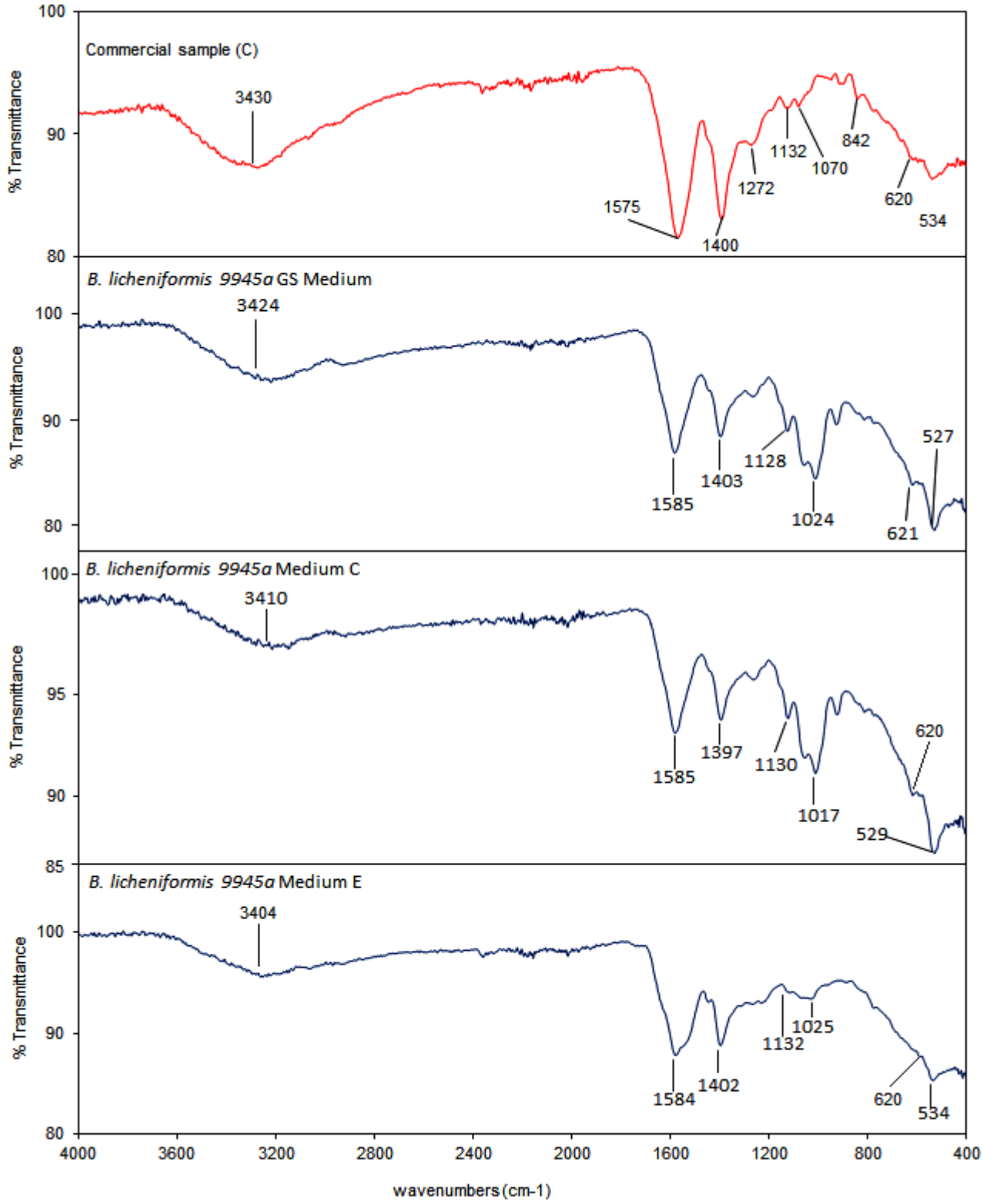


Figure 5.10: FT-IR spectra of γ-PGA(s) produced by *B. licheniformis* ATCC 9945a in GS, C and E media at 37°C and 150 rpm for 96 hours compared to the spectra of a commercial γ-PGA sample. Each spectrum is an average of 3 spectra (n=3)

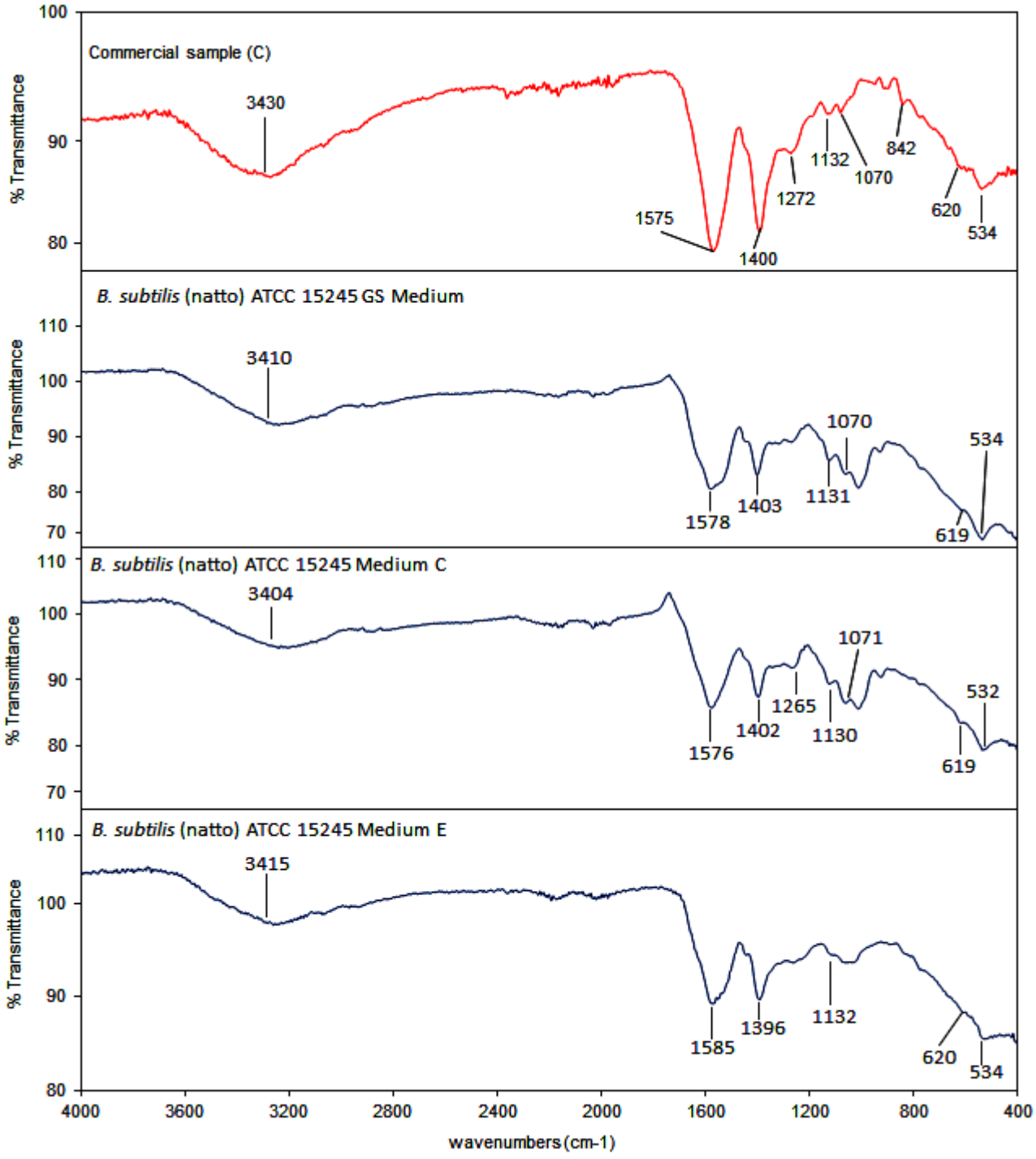


Figure 5.11: FT-IR spectra of γ-PGA(s) produced by *B. subtilis* (natto) ATCC 15245 in GS, C and E media at 37°C and 150 rpm for 96 hours compared to the spectra of a commercial γ-PGA sample. Each spectrum is an average of 3 spectra (n=3)

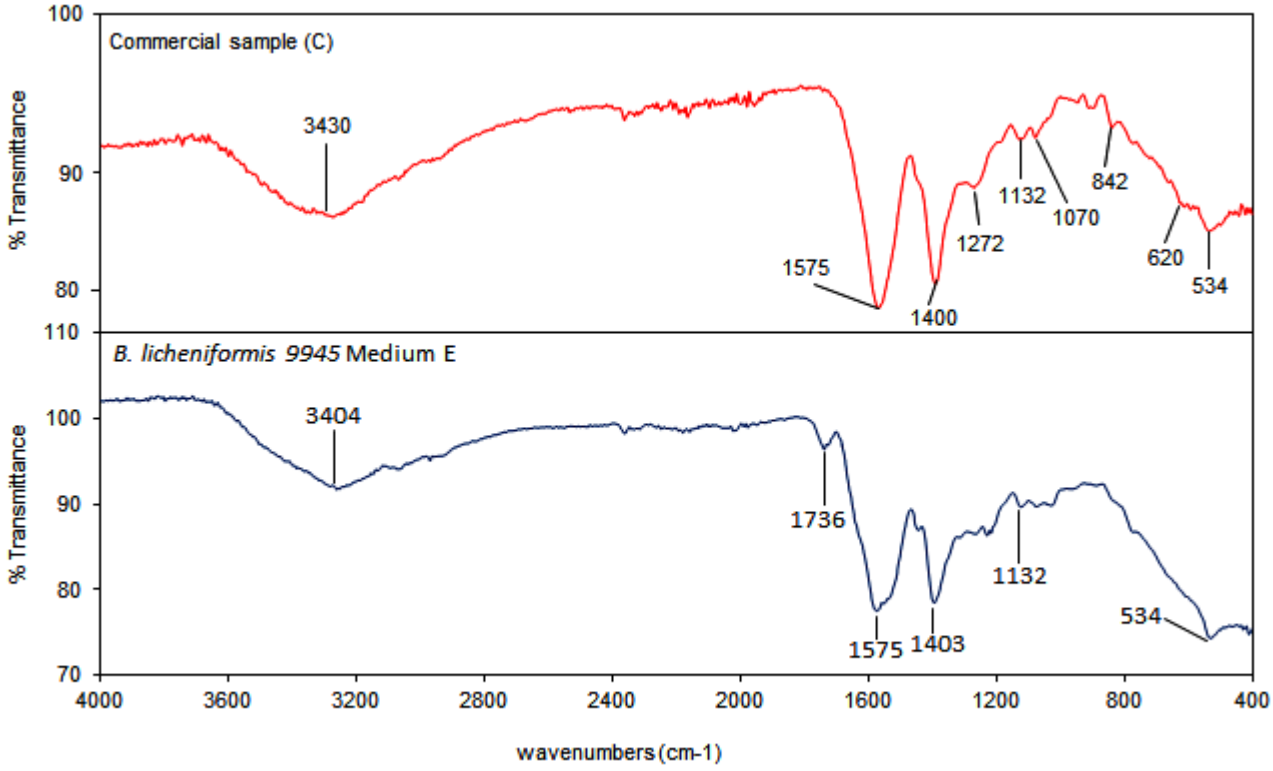


Figure 5.12: FT-IR spectrum of γ-PGA produced by *B. licheniformis* ATCC 9945 in medium E at 37°C and 150 rpm for 96 hours compared to the spectrum of a commercial γ-PGA sample. Spectrum is an average of 3 spectra (n=3)

5.7 Effects of temperature and time on γ-PGA production

As earlier mentioned, some *Bacillus* species such as *Bacillus coagulans* and *Bacillus licheniformis* have been reported to be thermophilic, growing at high temperature up to 70^{o}C (Abou-Dobara *et al*., 2011). Moreover, *Bacillus subtilis* NX-2 was found to produce a γ-PGA concentration of 30.2 g/l at 37^{o}C and 200 rpm after a short cultivation period of 24 hours in the fermentation medium (Xu *et al.*, 2005a). Since some *Bacillus* species are thermophilic and a strain of *Bacillus subtilis* - NX-2 has been reported to start secreting γ-PGA after only 4 hours of incubation until 24 hours when no more γ-PGA was produced in the medium, it was therefore necessary to investigate the production of γ-PGA at both 37^{o}C and 50^{o}C with respect to time. To assess the effects of different temperatures and times on the production of γ-PGA, *B. subtilis* (natto) ATCC 15245 was grown in GS medium and incubated aerobically at 37^{o}C and 50^{o}C and 150 rpm for a period of 96 hours. *B. subtilis* (natto) ATCC 15245 and GS medium were employed in the investigation of different temperatures and times on the production of γ-PGA because *B. subtilis* (natto) ATCC 15245 produced the maximum purified γ-PGA yield of 11.69 g/l when previously cultivated at 37^{o}C in GS medium for 96 hours (**Fig. 5.7**). Aliquots were taken at 24, 48, 72 and 96 hours and analysed for the extraction and recovery of possible γ-PGA or glutamic acid (**Table 5.2**).

Fig. 5.13 shows the comparison between the spectrum of the product obtained from the growth of *B. subtilis* (natto) ATCC 15245 in GS medium at 50^{o}C after 96 hours and that of glutamic acid. This indicates that at 50^{o}C, L-glutamic acid is not being utilized and converted into γ-PGA by *B. subtilis* (natto) ATCC 15245 because cell population starts to die just after 24 hours of incubation (**Fig. 5.5b**). Results (**Table 5.2**) show that at 37^{o}C, there was no production of γ-PGA until after 72 hours of cultivation. The yield of crude γ-PGA produced by *B. subtilis* (natto) ATCC 15245 at 72 hours was lower than that obtained at 96 hours suggesting that the L-glutamic acid in the culture medium has not been completely utilized and converted into γ-PGA at 72 hours.

As a result, further cultivation at 50°C was discontinued. The results confirmed the need to harvest γ-PGA at 96 hours and not earlier.

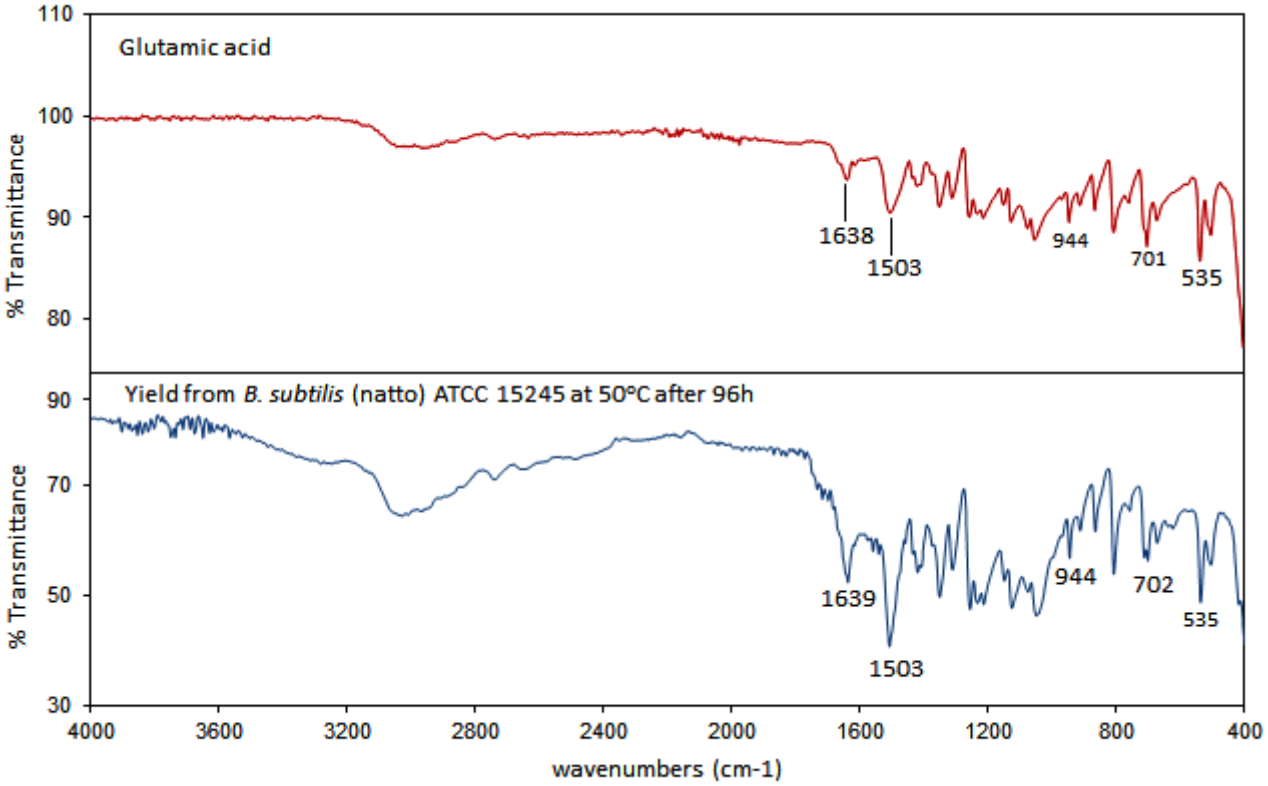


Figure 5.13: FT-IR spectrum of the product recovered when *B. subtilis* (natto) ATCC 15245 was cultured in GS medium at 50°C and 150 rpm for 96 hours compared to the spectrum of glutamic acid. Spectrum is an average of 3 spectra (n=3)

Table 5.2: Yields (mean ± SEM) of glutamic acid (GA)/γ-PGA with respect to growth time (24 -96 hours) and temperature (37°C and 50°C) by *B. subtilis* (natto) ATCC 15245 in GS medium. No γ-PGA was produced at 50°C and before 72 hours at 37°C (n=3)

Temperature	Time	Yield g/l (Mean ± SEM)	FTIR analysis	
			GA	γ-PGA
37°C	24	7.8 ± 0.49	+	-
	48	16.84 ± 0.098	+	-
	72	13.17 ± 0.23	-	+
	96	16.3 ± 0.34	-	+
50°C	24	11 ± 0.59	+	-
	48	16.62 ± 0.18	+	-
	72	17.5 ± 0.25	+	-
	96	15.14 ± 0.60	+	-

5.8 Analysis of γ-PGA using inductively coupled plasma spectrometry (ICP)

For an effective application of γ-PGA, it is important to analyse the γ-PGA elementally so as to know the form of the polymer before utilising it. It is also imperative to determine the percentage salt composition of the produced γ-PGA(s) since the solubility of γ-PGA depends on its form and to know if the bacterium and/or medium used in the production of γ-PGA influences its form. The solubility of γ-PGA is usually poor in solvents except for dimethyl sulfoxide (DMSO) and is only soluble in water when in alkali metal (e.g. sodium or potassium) salt form (Kubota *et al*., 1995).

The high yield polymers produced by different bacteria - *B. licheniformis* ATCC 9945a, *B. licheniformis* ATCC 9945 and *B. subtilis* (natto) ATCC 15245 in the three media were elementally analysed using ICP-AES to determine whether they are in salt or acid form **(Table 5.3)**.

Table 5.3: Percentage (mean ± SEM) sodium salt and acid composition of γ-PGA produced in (a) GS medium (b) Medium C and (c) Medium E at 37^{o}C and 150 rpm for 96 hours. Analysis was carried out using ICP-AES (SPECTRO CIROS^{CCD}) (n=3)

(a) GS medium

Strain name	% Na^{+}- γ-PGA	% H- γ-PGA
B. licheniformis ATCC 9945*a*	33.19 ± 0.27	47.81 ± 0.46
B. subtilis (natto) ATCC 15245	32.77 ± 0.70	48.53 ± 0.77

(b) Medium C

Strain name	% Na^{+}- γ-PGA	% H- γ-PGA
B. licheniformis ATCC 9945a	39.71 ± 0.13	45.58 ± 0.84
B. subtilis (natto) ATCC 15245	42.15 ± 0.25	43.42 ± 0.53

(c) Medium E

Strain name	% Na^{+}- γ-PGA	% H- γ-PGA
B. licheniformis ATCC 9945a	65.81 ± 1.16	31.19 ± 0.60
B. licheniformis ATCC 9945	56.17 ± 2.12	32.07 ± 1.13
B. subtilis (natto) ATCC 15245	56.90 ± 0.50	37.98 ± 1.04

Results showed that the γ-PGA(s) produced by *B. licheniformis* ATCC 9945a and *B. subtilis* (natto) ATCC 15245 in GS medium and medium C were more of the acid form of γ-PGA (H-γ-PGA) than the sodium salt form (Na^+-γ-PGA). In contrast, the γ-PGA(s) produced by bacterial strains grown in medium E were mostly the sodium salt form of γ-PGA.

5.9 Molecular weight of γ-PGA

The molecular weight is another important feature of γ-PGA that needs to be investigated. γ-PGA can have molecular weights ranging from ≈10 kDa to greater than 1,000 kDa (Bajaj *et al*., 2009). The application of γ-PGA is to a large extent dependent on its molecular weight. Since γ-PGA is produced microbially, its molecular weight is highly dependent on factors including the bacterial strain used, culture conditions, media composition and the action of enzymes excreted by the microbe in the late stationary phase of growth (Candela and Fouet, 2006). Assessing the molecular weight of γ-PGA will give an understanding as to whether the molecular weight of γ-PGA depends on the bacterium and/or medium used in producing the polymer.

In addition to molecular weight, dispersity index (*Đ*) is also an essential characteristic of γ-PGA because the nearer the value of *Đ* is to unity, the more the homogeneity of the γ-PGA is as regards its molecular weight. **Table 5.4** shows the molecular weight and *Đ* of γ-PGA(s) produced by *B. licheniformis* ATCC 9945a, *B. licheniformis* ATCC 9945 and *B. subtilis* (natto) ATCC 15245 in three media as analysed by aqueous based GPC.

Table 5.4: Molecular weight and dispersity of γ-PGA produced by different bacteria in (a) GS medium (b) Medium C and (c) Medium E at 37°C and 150 rpm for 96 hours. Analysis was carried out using GPC (PL aquagel-OH guard plus 2 * PL aquagel-OH MIXED-H)

(a) GS medium

Strain name	Molecular weight (Da) (M_w)	Molecular number (M_n)	Dispersity (M_w/M_n)
B. licheniformis ATCC 9945a	1,420,000	970,000	1.5
B. subtilis (natto) ATCC 15245	1,470,000	1,070,000	1.4
**B. subtilis (natto) ATCC 15245*	257,500	54550	4.75

(b) Medium C

Strain name	Molecular weight (Da) (M_w)	Molecular number (M_n)	Dispersity (M_w/M_n)
B. licheniformis ATCC 9945a	1,620,000	1,020,000	1.6
B. subtilis (natto) ATCC 15245	1,650,000	1,100,000	1.5

(c) Medium E

Strain name	Molecular weight (Da) (M_w)	Molecular number (M_n)	Dispersity (M_w/M_n)
B. licheniformis ATCC 9945a	753,000	519,000	1.4
B. licheniformis ATCC 9945	779,000	514,000	1.5
B. subtilis (natto) ATCC 15245	760,000	605,000	1.3

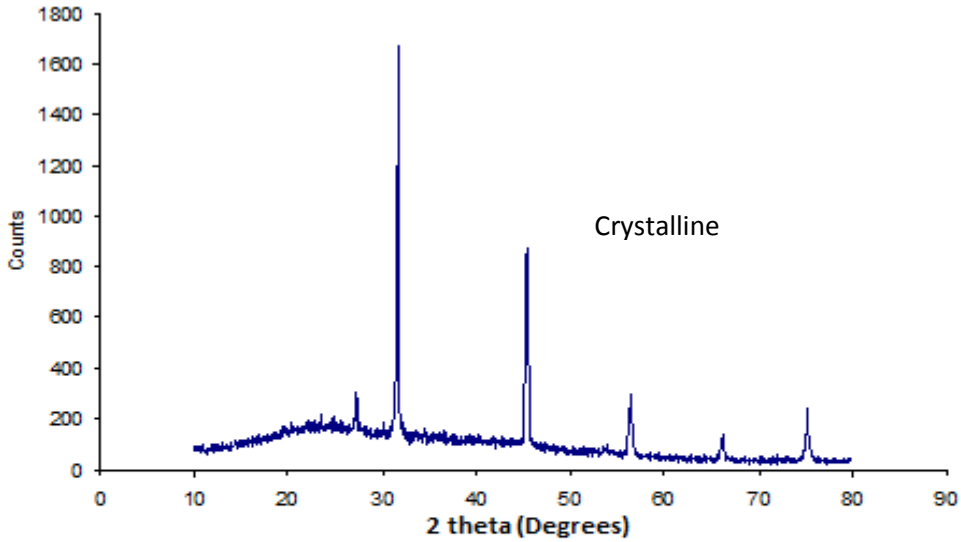
* γ-PGA produced from GS medium containing 50g/l NaCl

In both GS medium and medium C, *B. licheniformis* ATCC 9945a and *B. subtilis* (natto) ATCC 15245 produced γ-PGA(s) with similar molecular weights of ≈ 1,400 k Da and ≈ 1,600 k Da from GS and C media respectively whereas in medium E, they produced γ-PGA(s) with lower molecular weights (≈ 750 k Da). γ-PGA from *B. licheniformis* ATCC 9945 which only produced biopolymer in medium E had a molecular weight similar to those of γ-PGA(s) from *B. licheniformis* ATCC 9945a and *B. subtilis* (natto) ATCC 15245 also in medium E (**Table 5.4c**).

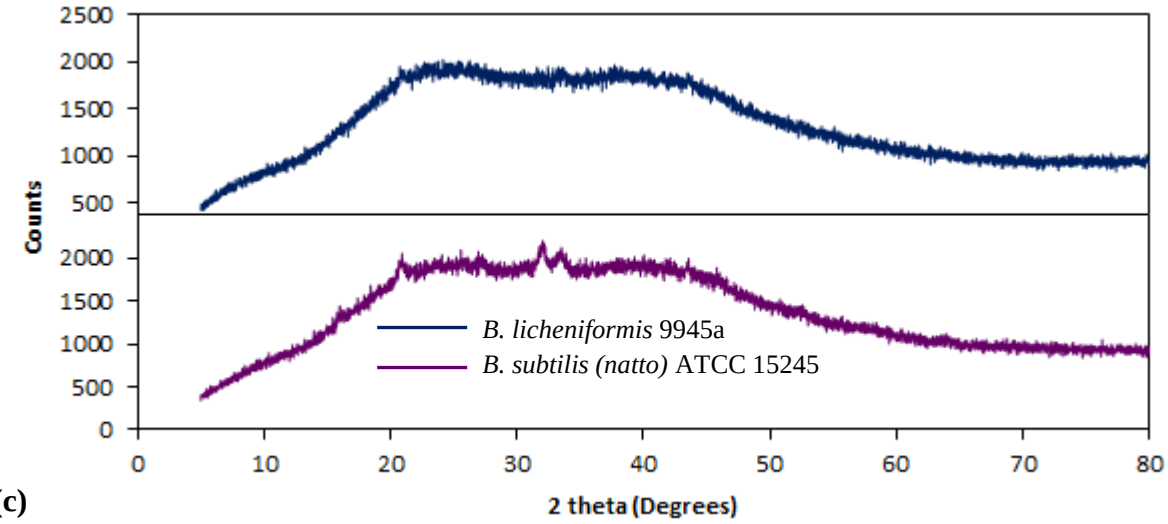
5.10 Crystallinity of γ-PGA

Like the molecular weight and salt composition of γ-PGA, crystallinity is also an essential characteristic of γ-PGA. Crystallinity describes the long range order at the atomic level within a polymer (Rausch, 2009). The crystallinity of a polymer affects its characteristics such as tensile strength, opacity, melting point and solubility. A polymer can be completely amorphous having no long range order (no crystallinity) or have some degree of crystallinity. Polymer crystallinity plays an important role in determining its solubility behaviour (Rausch, 2009). The more crystalline a material is, the harder the material is and the higher its density (Wainwright *et al*., 1982). On the other hand, amorphous polymers are softer and are easily penetrated by solvents (Wainwright *et al*., 1982 and Schultz, 2013). Furthermore, the application for which γ-PGA is being investigated requires a water-soluble γ-PGA. Thus, it is important to analyse the produced γ-PGA(s) to determine if they are crystalline or amorphous. The degree of crystallinity of γ-PGA(s) produced by *B. licheniformis* ATCC 9945a, *B. licheniformis* ATCC 9945 and *B. subtilis* (natto) ATCC 15245 in three media were analysed using XRD. **Fig. 5.14** represents the XRD spectra of commercial γ-PGA samples and these γ-PGA(s) produced after growth in media GS, C and E respectively.

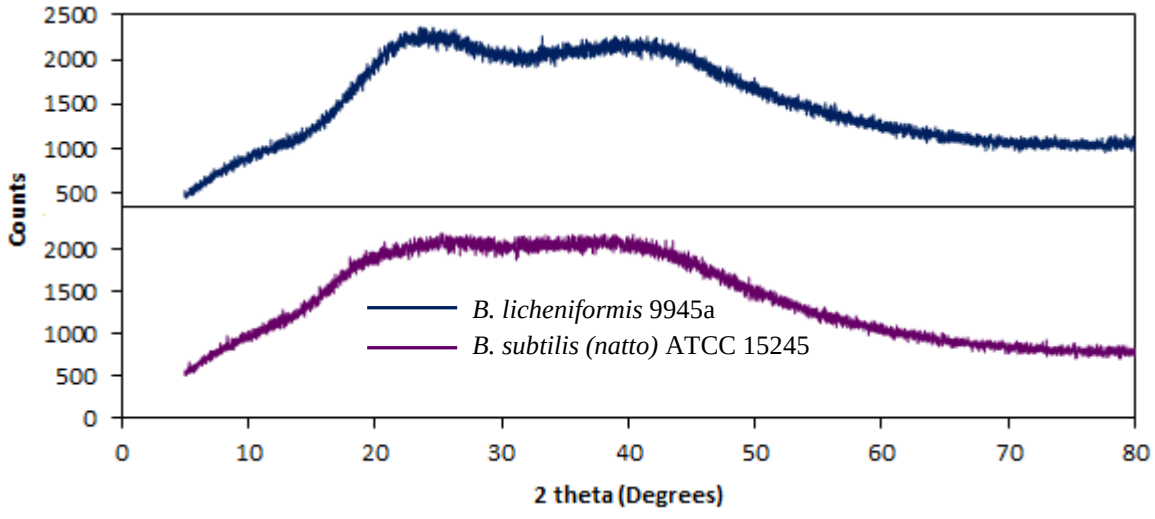
(a)


Counts
Crystalline
2 theta (Degrees)
Amorphous
Counts
2 theta (Degrees)

(b)


Counts
B. licheniformis 9945a
B. subtilis (natto) ATCC 15245
2 theta (Degrees)

(c)


Counts
B. licheniformis 9945a
B. subtilis (natto) ATCC 15245
2 theta (Degrees)

(d)

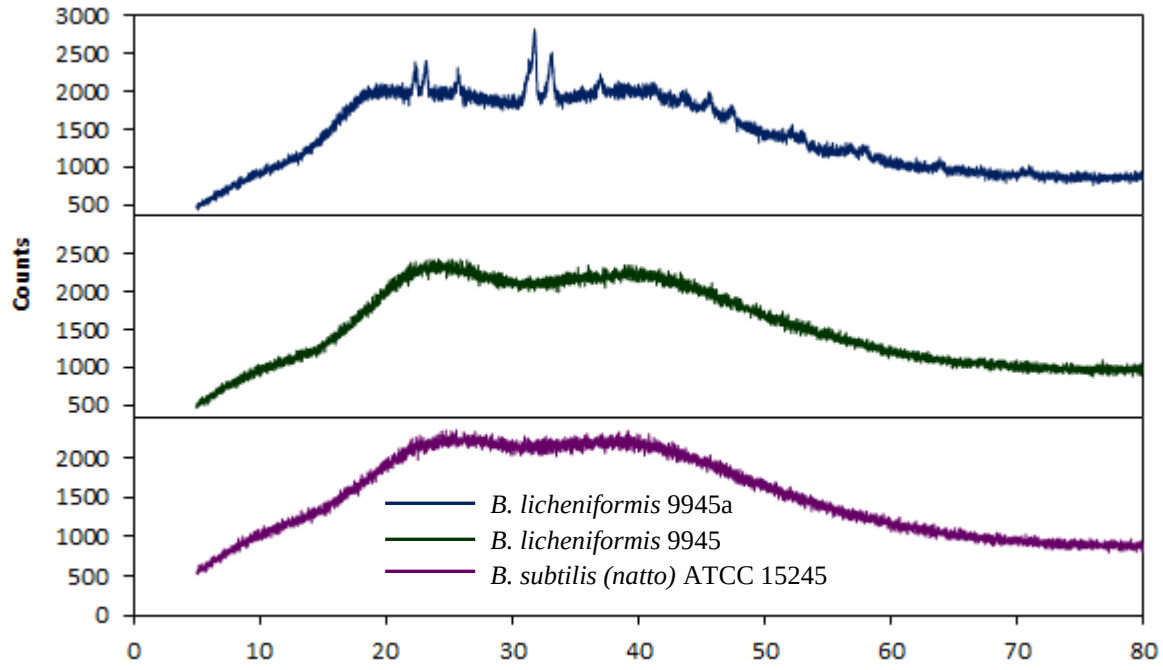


Figure 5.14: Crystallinity of (a) commercial γ-PGA samples and γ-PGA produced by *B. licheniformis* ATCC 9945a and *B. subtilis* (natto) ATCC 15245 in (b) GS medium and (c) medium C) at 37°C and 150 rpm for 96 hours (d) Crystallinity of γ-PGA produced by *B. licheniformis* ATCC 9945 in medium E at 37°C and 150 rpm for 96 hours. Spectrum is an average of 3 spectra (n=3)

The broad peaks seen in the XRD spectra of γ-PGAs produced by *B. licheniformis* ATCC 9945a and *B. subtilis* (natto) ATCC 15245 in GS medium and medium C (**Fig. 5.14b & c**) are characteristic of amorphous γ-PGA (**Fig. 5.14a**). The XRD spectra of γ-PGAs produced by *B. licheniformis* ATCC 9945 and *B. subtilis* (natto) ATCC 15245 in medium E are also indicative of amorphous γ-PGA (**Fig. 5.14d**) while the XRD spectrum of γ-PGA produced by *B. licheniformis* ATCC 9945a in medium E appears to be semi-crystalline, having small crystalline regions (sharp and narrow diffraction peaks) at 26, 32, 33 and 37 degrees of 2 theta (θ) in-between regions of amorphous polymer (**Fig. 5.14d)**.

5.11 Summary of bacterial production of γ-PGA

The production of γ-PGA by five *Bacilli* strains in GS medium, medium C and medium E at 37 and 50^{o}C and 150 rpm for a period 96 hours was investigated. The results are summarised in **Table 5.5**. It was observed that each strain produced γ-PGA extracellularly when grown aerobically in one or all three media at 37^{o}C (only) and 150 rpm for 96 hours. Results showed that polymers produced by four out of the five investigated bacteria - *Bacillus licheniformis* 1525, *Bacillus licheniformis* NCTC 6816, *B. subtilis* (natto) ATCC 15245 and *B. licheniformis* ATCC 9945a from GS medium and medium C were off-white in colour while those obtained from medium E by all investigated bacteria were brownish amorphous products. The produced polymers were identified as γ-PGA using FT-IR analysis. It was discovered that crystallinity of γ-PGA depended on the medium of production while its molecular weight was seen to be influenced by both the medium of production and the bacterial strain it.

After characterizing γ-PGA based on its yield, form, crystallinity and molecular weight, *B. subtilis* (natto) ATCC 15245 was chosen for further production of γ-PGA to be used for heavy metal removal application. *B. subtilis* (natto) ATCC 15245, which produced the highest yield of γ-PGA in both GS medium (11.69 g/l) and medium C (11.59 g/l), secreted the highest molecular weight γ-PGA (M_w 1,650,000 Da) in medium C, is non-toxic and completely soluble in water makes its γ-PGA appropriate for heavy metal removal application since particularly a high molecular weight γ-PGA is necessary for applications in water and wastewater treatment (Bajaj and Singhal, 2011b). Since γ-PGA produced by *B. subtilis* (natto) ATCC 15245 in medium C was preferred, only that γ-PGA was analysed by NMR to further confirm its structure. **Fig. 5.15** shows the proton NMR spectra of γ-PGA obtained from *B. subtilis* (natto) ATCC 15245 in medium C and a commercial sample. The spectrum of γ-PGA produced by *B. subtilis* (natto) ATCC 15245 shows similar peaks to that of the commercial γ-PGA.

Table 5.5: Summary of γ-PGA (mean ± SEM) produced by 5 *Bacillus* species in (a) GS medium (b) Medium C and (c) Medium E at 37°C and 150 rpm for 96 hours (n=3)

(a) GS medium

Organism	Yield (g/l)	% Na- γ-PGA	% H^+- γ-PGA	Molecular weight (Da)	No. of glutamic acid monomers
B. licheniformis 1525	4.52 ± 0.04	n/a	n/a	n/a	n/a
B. licheniformis NCTC 6816	4.45 ± 0.09	n/a	n/a	n/a	n/a
B. licheniformis ATCC 9945a	10.95 ± 1.26	33.19 ± 0.27	47.81 ± 0.46	1,420,000 ($Đ$ = 1.5)	~9,651
B. licheniformis ATCC 9945	0	n/a	n/a	n/a	n/a
B. subtilis (natto) ATCC 15245	11.69 ± 0.27	32.77 ± 0.70	48.53 ± 0.77	1,470,000 ($Đ$ = 1.4)	~9,991

(b) Medium C

Organism	Yield (g/l)	% Na- γ-PGA	% H^+- γ-PGA	Molecular weight (Da)	No. of glutamic acid monomers
B. licheniformis 1525	4.55 ± 0.03	n/a	n/a	n/a	n/a
B. licheniformis NCTC 6816	4.44 ± 0.04	n/a	n/a	n/a	n/a
B. licheniformis ATCC 9945a	11.37 ± 0.66	39.71 ± 0.13	45.58 ± 0.84	1,620,000 ($Đ$ = 1.6)	~11,011
B. licheniformis ATCC 9945	0	n/a	n/a	n/a	n/a
B. subtilis (natto) ATCC 15245	11.59 ± 0.40	42.15 ± 0.25	43.42 ± 0.53	1,650,000 ($Đ$ = 1.5)	~11,215

(c) Medium E

Organism	Yield (g/l)	% Na- γ-PGA	% H^+- γ-PGA	Molecular weight (Da)	No. of glutamic acid monomers
B. licheniformis 1525	4.08 ± 0.02	n/a	n/a	n/a	n/a
B. licheniformis NCTC 6816	4.05 ± 0.11	n/a	n/a	n/a	n/a
B. licheniformis ATCC 9945a	10.27 ± 0.41	65.81 ± 1.16	31.19 ± 0.60	753,000 ($Đ$ = 1.4)	~5,112
B. licheniformis ATCC 9945	8.73 ± 0.22	56.17 ± 2.12	32.07±1.13	779,000 ($Đ$ = 1.5)	~5,295
B. subtilis (natto) ATCC 15245	6.04 ± 0.28	56.90 ± 0.50	37.98 ± 1.04	760,000 ($Đ$ = 1.3)	~5,166

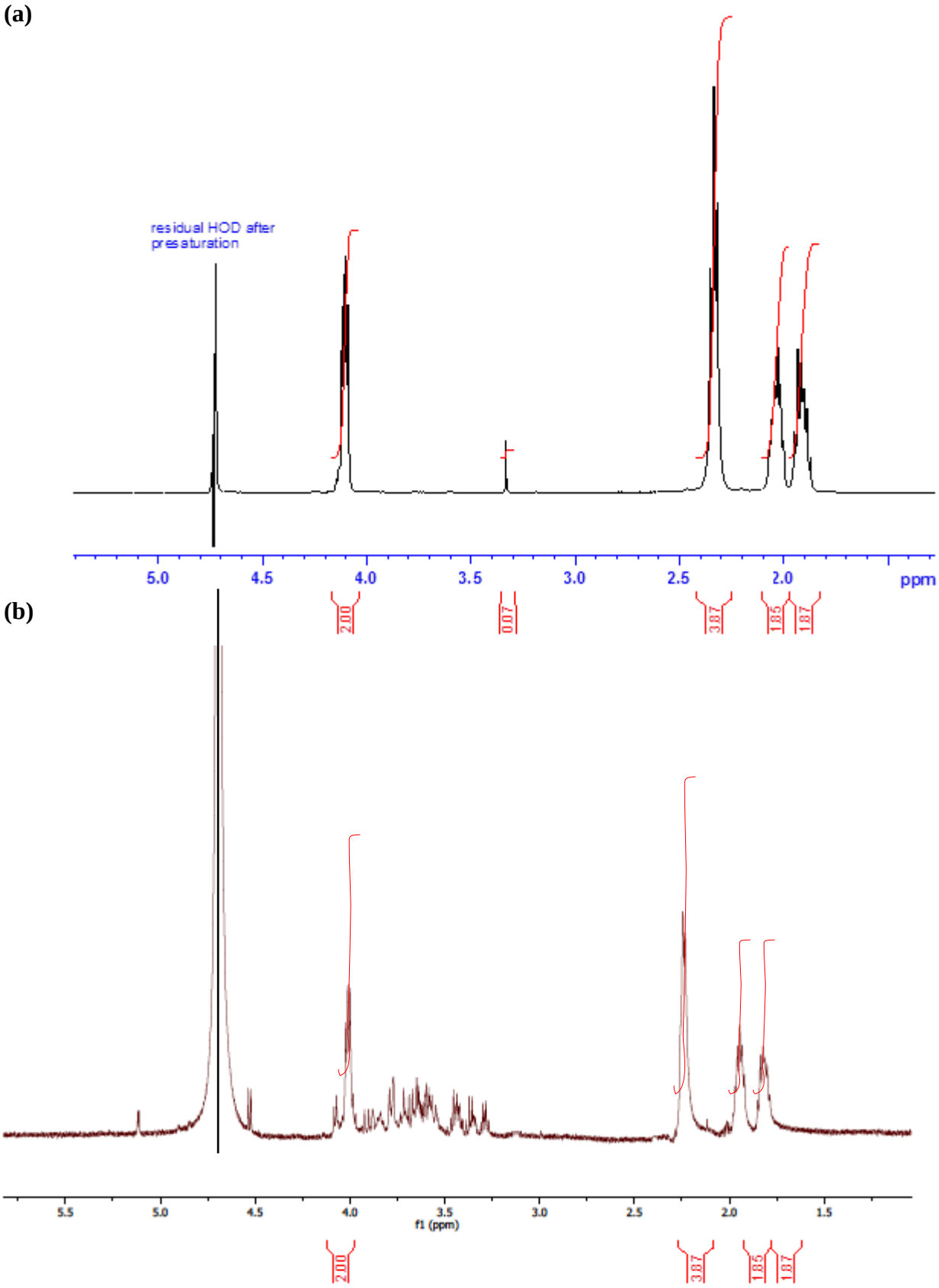


Figure 5.15: ^{1}H-NMR spectra of (a) commercial γ-PGA sample and (b) γ-PGA produced by *B. subtilis* (natto) ATCC 15245 in medium C

6.0 RESULTS – REMOVAL OF HEAVY METALS BY POLY-GAMMA-GLUTAMIC ACID (γ-PGA)

6.1 Introduction

The results of the investigations regarding the batch adsorption of Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, and Ag^{+} from synthetic wastewater using γ-PGA as adsorbent and the effects of process variables such as pH, γ-PGA concentration and other competing metal ions are presented in this chapter. γ-PGA was produced by *B. subtilis* (natto) ATCC 15245 (M_w 1,650,000 Da) in medium C at 37^{o}C and 150 rpm for 96 hours. The polymer was isolated after 96 hours, purified, identified, characterized and then was used for the removal of heavy metals. The adsorption capability of this polymer was tested with all metal ions (Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, and Ag^{+}) under study. Sorption isotherm models were applied to investigate the removal performance. ICP was used to analyse the metal concentration in the prepared wastewater before and metal adsorption by γ-PGA. All experiments were carried out in triplicates and the results described here are the average of three sets of experiments.

6.2 Effect of γ-PGA concentration on metal ion adsorption

Adsorbent dose plays an important role in the process of adsorption since the more available active binding sites or surface area there is for adsorption, the higher the percentage removal of the sorbate (in this case metal ions) (El-Sayed and El-Sayed, 2014). The effect of γ-PGA dose on removal of Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, and Ag^{+} was studied by varying the concentration of γ-PGA from 0.1 to 1.0 g/l at room temperature (25^{o}C/298 K) in a non-pH controlled system. The results are presented in **Fig. 6.1**. Results showed that the percentage of metal ions removal increased from 41.45% to 93.50%, 12.26% to 88.13%, 30.50% to 90.21%, 18.76% to 90.56% and 38.09% to 86.34% when γ-PGA concentration was increased from 0.1 to 1.0 g/l for Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+} and Ag^{+} respectively. The percentage removal of metal ions increased with increasing γ-PGA concentration which can be attributed to the presence of more adsorption sites at higher concentrations of γ-PGA.

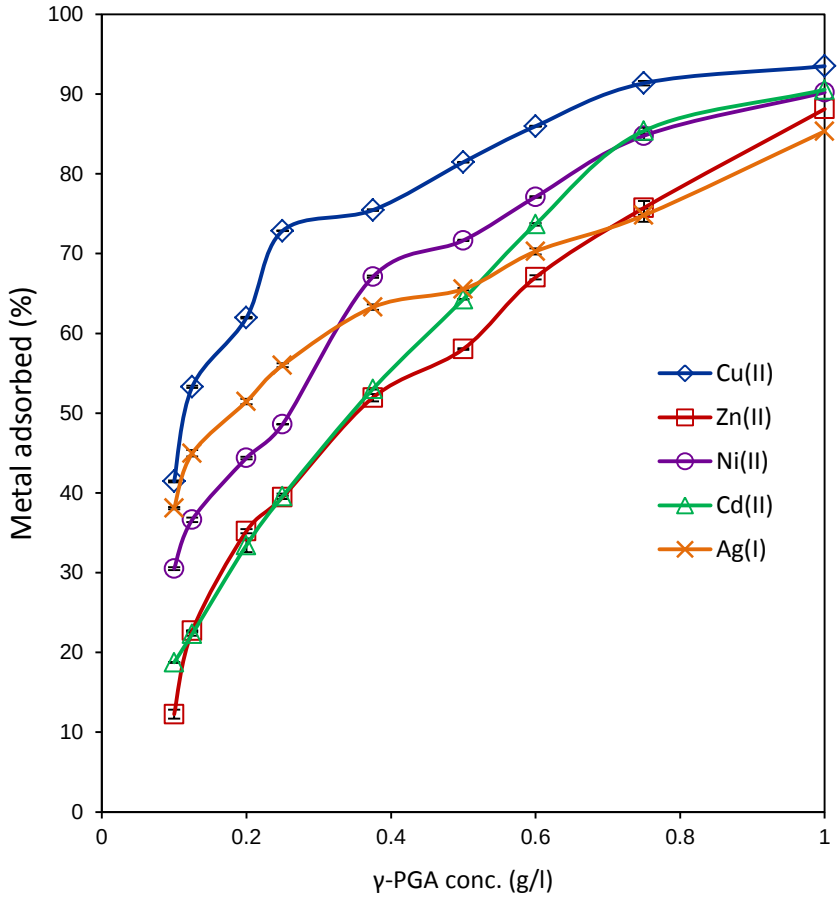


Figure 6.1: Effect of γ-PGA concentration on adsorption of heavy metal ions (mean ± SEM). Adsorption experiments: metal concentration, 50mg/l; γ-PGA concentration, 0.1 - 1.0 g/l; Time, 12 hours; mean (n=3)

6.3 Effect of pH on metal ion adsorption

The pH of the solution is also an important factor that determines metal adsorption since the mechanism of adsorption involves the binding of solutes by sorbents (Inbaraj *et al.*, 2009). Moreover, assessing the effect of pH on the adsorption of heavy metals by γ-PGA which is a polymer with several charged surface functional groups will be of great significance as pH has the ability to affect the dissociation of cation exchange groups and conformation of γ-PGA as well as stability of the metal complexes formed with γ-PGA (Inbaraj *et al.*, 2009). Analysis of the solution by ICP-AES will determine if solution pH influences heavy metals adsorption by γ-PGA or not. **Fig. 6.2** illustrates the effect of solution pH on the adsorption of (50mg/l) Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+} and Ag^{+} by γ-PGA. The maximum pH values selected for the

adsorption of Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+} and Ag^{+} were 6.0, 7.0, 7.5, 8.0 and 7.5 respectively in order to prevent the precipitation of their metal hydroxide.

No adsorption took place in all metal ions until pH values reached 3. Metal adsorption then increased from 2.98% to 22.36% for Cu^{2+} at pH 6.0, 0.89% to 62.64% for Zn^{2+} at pH 7.0, 13.33% to 62.80% for Ni^{2+} at pH 7.5, 1.63% to 61.03% for Cd^{2+} at pH 8.0 and 2.17% to 53.15% for Ag^{+} at pH 7.5. Results showed that even though the amount of metal ion adsorbed increased with increasing pH values before metal ions started to precipitate, the adsorption capacity of γ-PGA in a non pH controlled system (**Fig. 6.1**) was higher than in a pH controlled system (**Fig.6.2**).

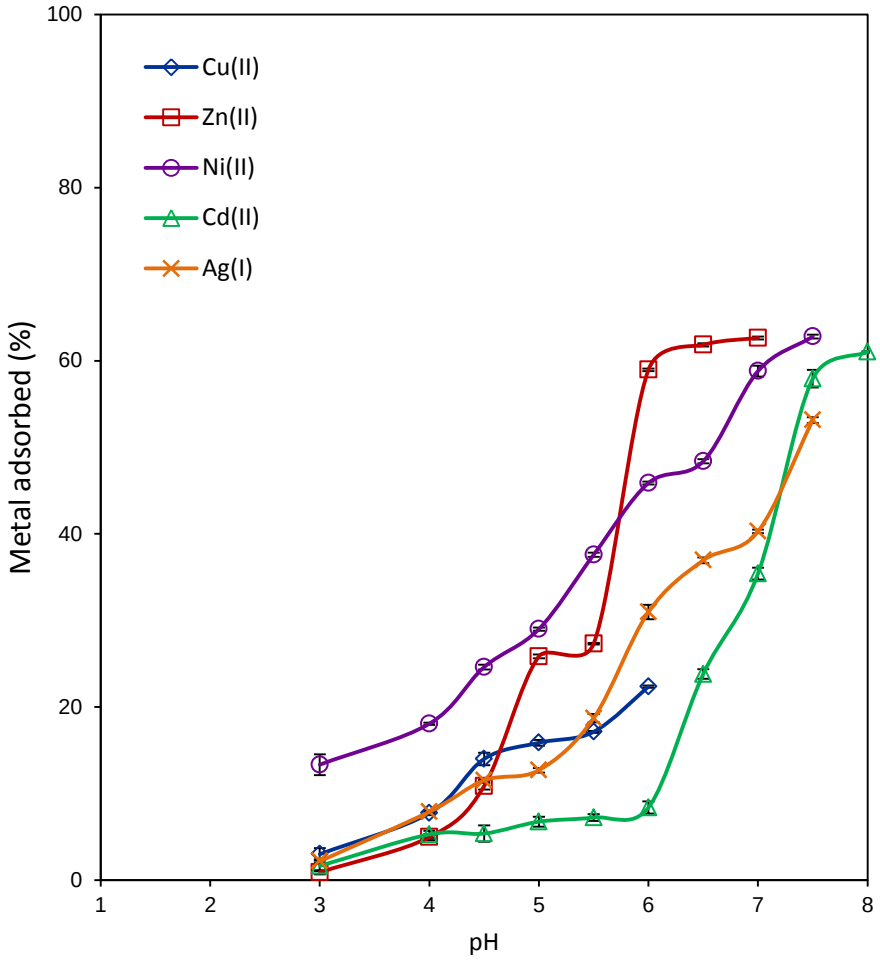


Figure 6.2: Effect of solution pH on adsorption of heavy metal ions (mean ± SEM). Adsorption experiments: γ-PGA concentration, 1.0 g/l; metal concentration, 50mg/l; pH range, 3-8; Time, 12 hours; mean (n=3)

6.4 Effect of competing heavy metal ions on individual metal ion adsorption

The effect of the mixture of all divalent heavy metal ions under study (Cu^{2+} + Zn^{2+} + Ni^{2+} + Cd^{2+}) on the adsorption of each of the metal ions was investigated to assess the selectivity of heavy metal adsorption (**Fig. 6.3**). This is important in determining the possibility of using an adsorbent for the recovery of a particular target metal ion (Inbaraj *et al*., 2009). This experiment was carried out at pH 5.5 to avoid the precipitation of metal ions.

From **Fig. 6.3**, it can be seen that when γ-PGA concentration was increased from 0.1 to 1.0 g/l, percentage metal adsorption slightly increased from 27.22 to 29.87%, 21.95 to 26.53%, 16.07 to 21.94 and 12.23 to 19.60% for Cu^{2+}, Zn^{2+}, Ni^{2+} and Cd^{2+} respectively. The affinities of the metal ions for γ-PGA increased in the order: Cu^{2+} > Zn^{2+} > Ni^{2+} > Cd^{2+}.

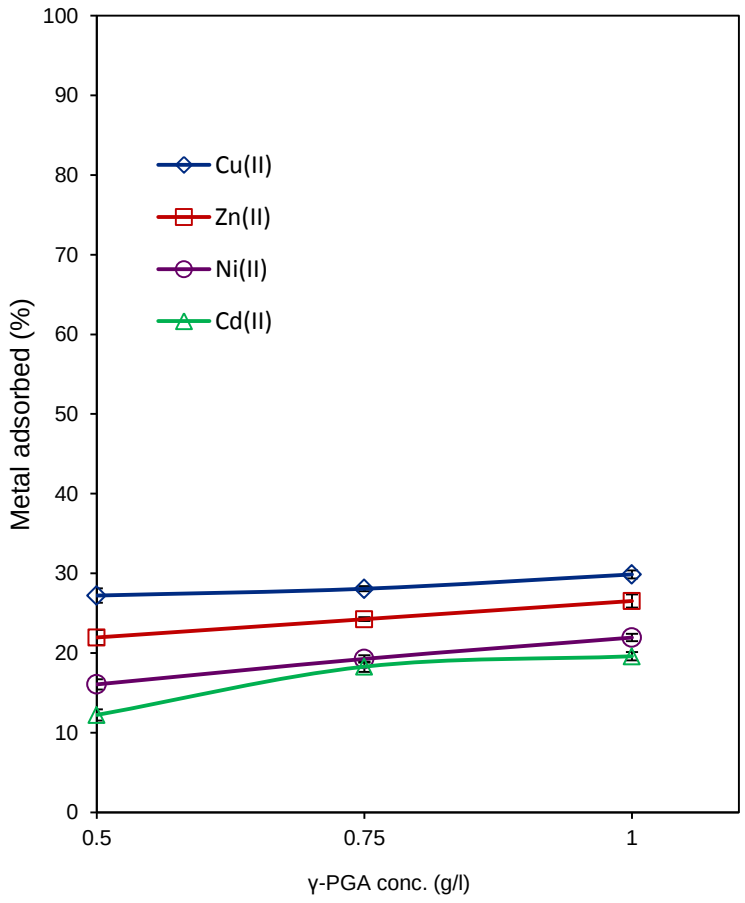


Figure 6.3: Effect of competing heavy metal ions on adsorption of individual heavy metal ion (mean ± SEM). Adsorption experiments: γ-PGA concentration, 0.5 - 1.0 g/l; metal concentration, 50mg/l; pH 5.5; Time, 12 hours; mean (n=3)

6.5 Effect of molecular weight on metal ion adsorption

The molecular weight of γ-PGA is an important characteristic on which its application depends (Ogunleye *et al*., 2015). As earlier mentioned, γ-PGAs of different molecular weights are required for different applications. For example, a low molecular weight (50 k Da) γ-PGA stimulates natural killer dendritic cells to produce cytokines and eliminate tumour cells (Lee *et al*., 2014) while a higher molecular weight (990 k Da) γ-PGA is necessary for applications in water and wastewater treatment (Bajaj and Singhal, 2011b). The effect of molecular weight on the removal of heavy metal ion was studied by varying the concentration of LMW and HMW γ-PGA (0.1 to 1.0 g/l) for the adsorption of Cu^{2+}. Cu^{2+} was selected for investigation since the adsorption capacity of γ-PGA was highest for Cu^{2+} amongst the investigated metal ions. The results are presented in **Fig. 6.4.** From **Fig. 6.4**, the percentage removal of removal of Cu^{2+} increased from 11.26% to 59.48% and 41.45% to 93.50% for LMW and HMW γ-PGA respectively. Results showed that the molecular weight of γ-PGA influences its adsorption capacity.

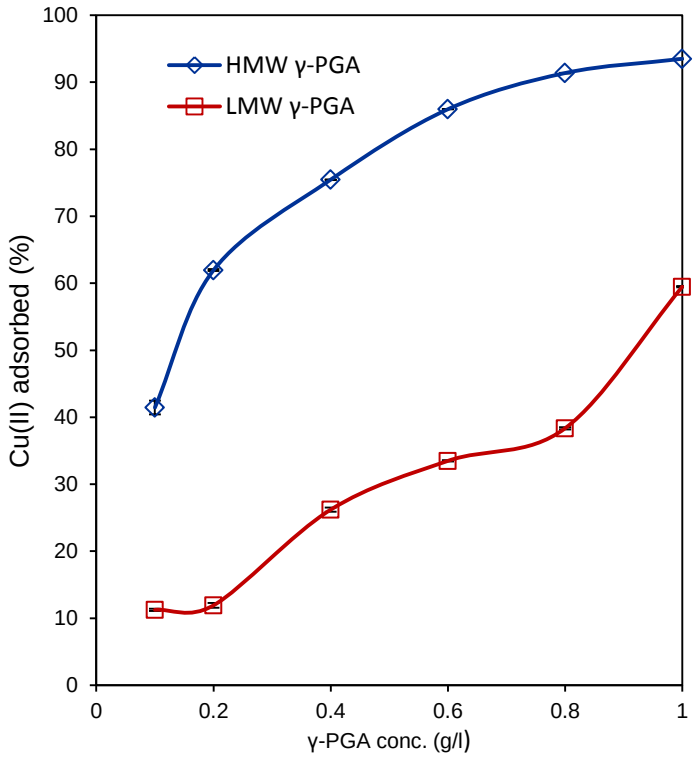


Figure 6.4: Effect of molecular weight on adsorption of heavy metal ions (mean ± SEM). Adsorption experiments: Cu^{2+} concentration, 50mg/l; γ-PGA concentration, 0.1 - 1.0 g/l; Time, 12 hours; mean (n=3)

6.6 Sorption isotherms

Adsorption isotherms are fundamental criteria required for describing an adsorption system. The equilibrium data are important in optimizing the design variables for any adsorption process (Inbaraj *et al*., 2006). An adsorption isotherm defines the relationship between the mass (q) of the solute (heavy metal ion) adsorbed per unit mass of adsorbent (γ-PGA) and the solute concentration in the solution at equilibrium (C_e) (Foo and Hameed, 2010).

Figs. 6.5 to 6.9 present the adsorption isotherms of Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+} and Ag^{+} by γ-PGA. Adsorption studies were conducted at fixed initial heavy metals concentration (50 mg/l) and room temperature (25^{o}C/298 K) by varying γ-PGA concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 g/l). Three equilibrium isotherms - Langmuir, Freundlich and Redlich-Peterson isotherms were tested for the adsorption of heavy metal ions onto γ-PGA to evaluate which model best fits the experimental data. The derived parameters of each isotherm model and their resulting error functions are presented in **Table 6.1**.

Fig. 6.5 to **6.9** represents the amount of metal ions adsorbed at room temperature. The isotherm data obtained indicated that the maximum heavy metal adsorption capacity of γ-PGA obtained for Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+} and Ag^{+} were 207.23 mg/g, 90.8 mg/g, 152.51 mg/g, 93.79 mg/g and 190.44 mg/g respectively.

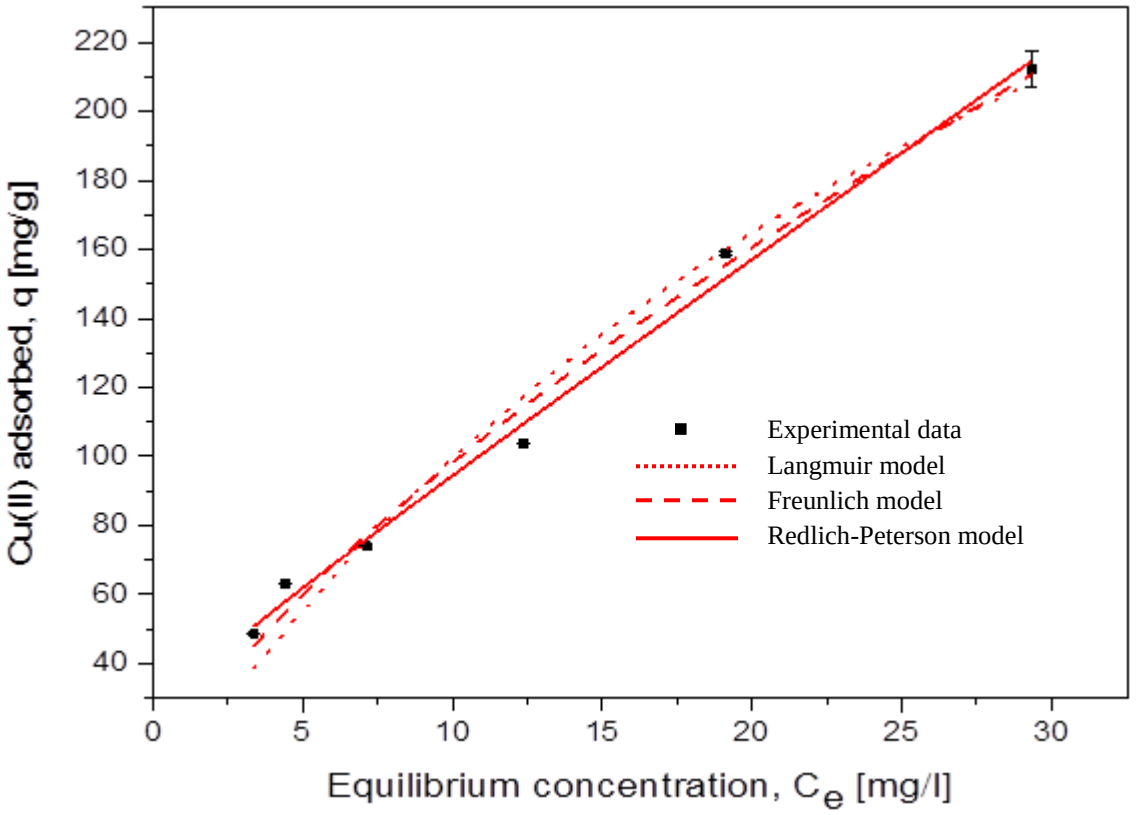


Figure 6.5: Adsorption isotherms with fitted for copper (II) adsorption on γ-PGA at room temperature (25°C/298 K). γ-PGA concentration range: 0.1-1.0 g/l; copper concentration: 50 mg/l; equilibrium time: 12 hours (mean ± SD) n=3.

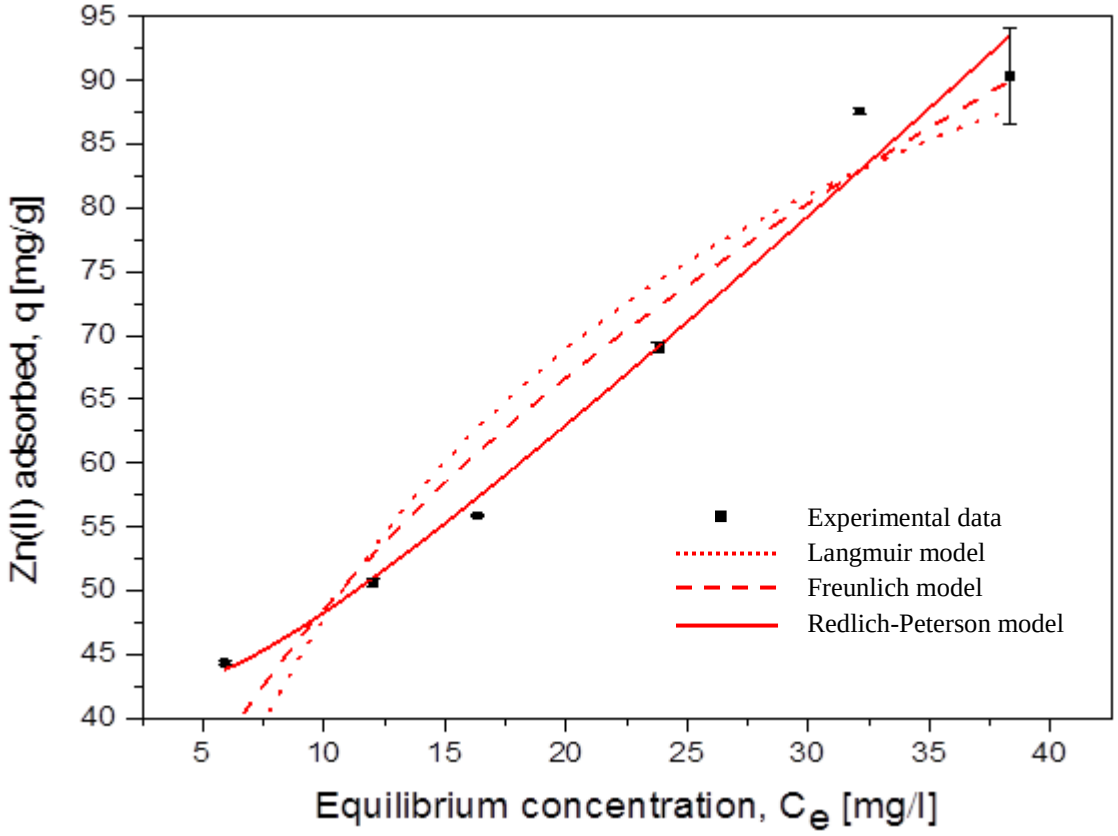


Figure 6.6: Adsorption isotherms with fitted for zinc (II) adsorption on γ-PGA at room temperature (25°C/298 K). γ-PGA concentration range: 0.1-1.0 g/l; zinc concentration: 50 mg/l; equilibrium time: 12 hours (mean ± SD) n=3.

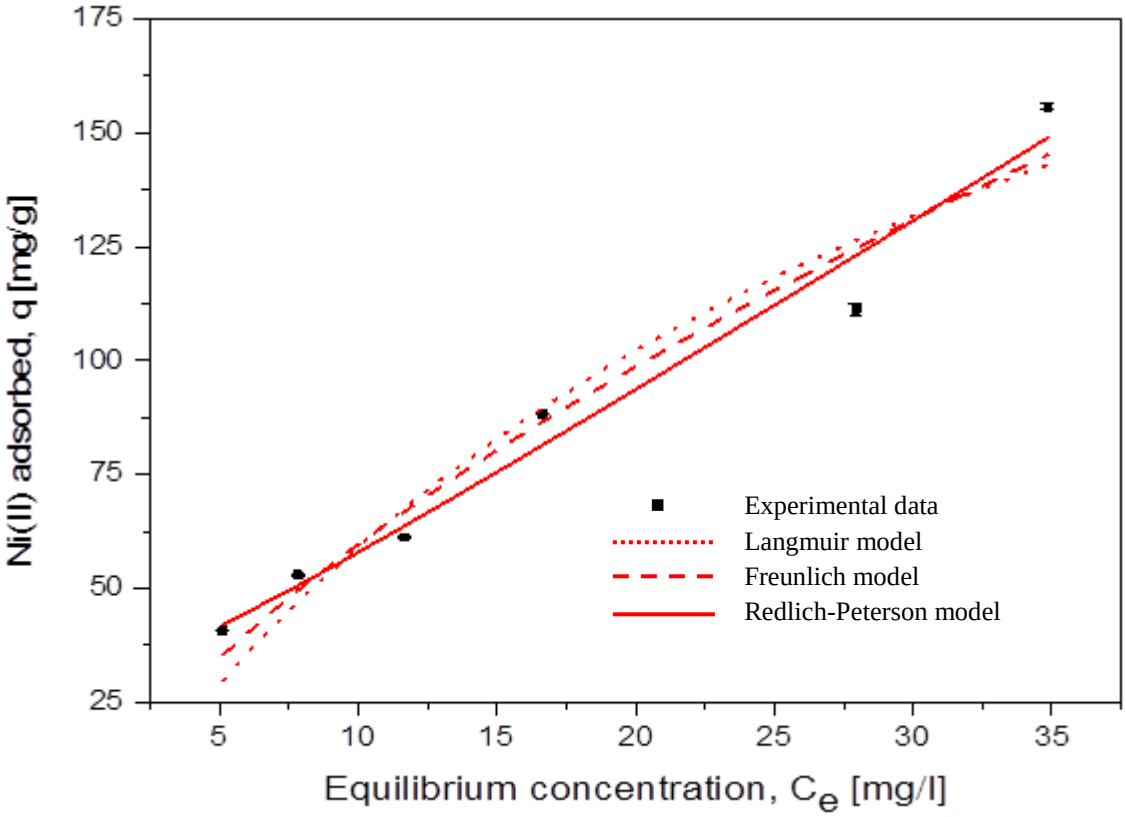


Figure 6.7: Adsorption isotherms with fitted for nickel (II) adsorption on γ-PGA at room temperature (25°C/298 K). γ-PGA concentration range: 0.1-1.0 g/l; copper concentration: 50 mg/l; equilibrium time: 12 hours (mean ± SD) n=3.

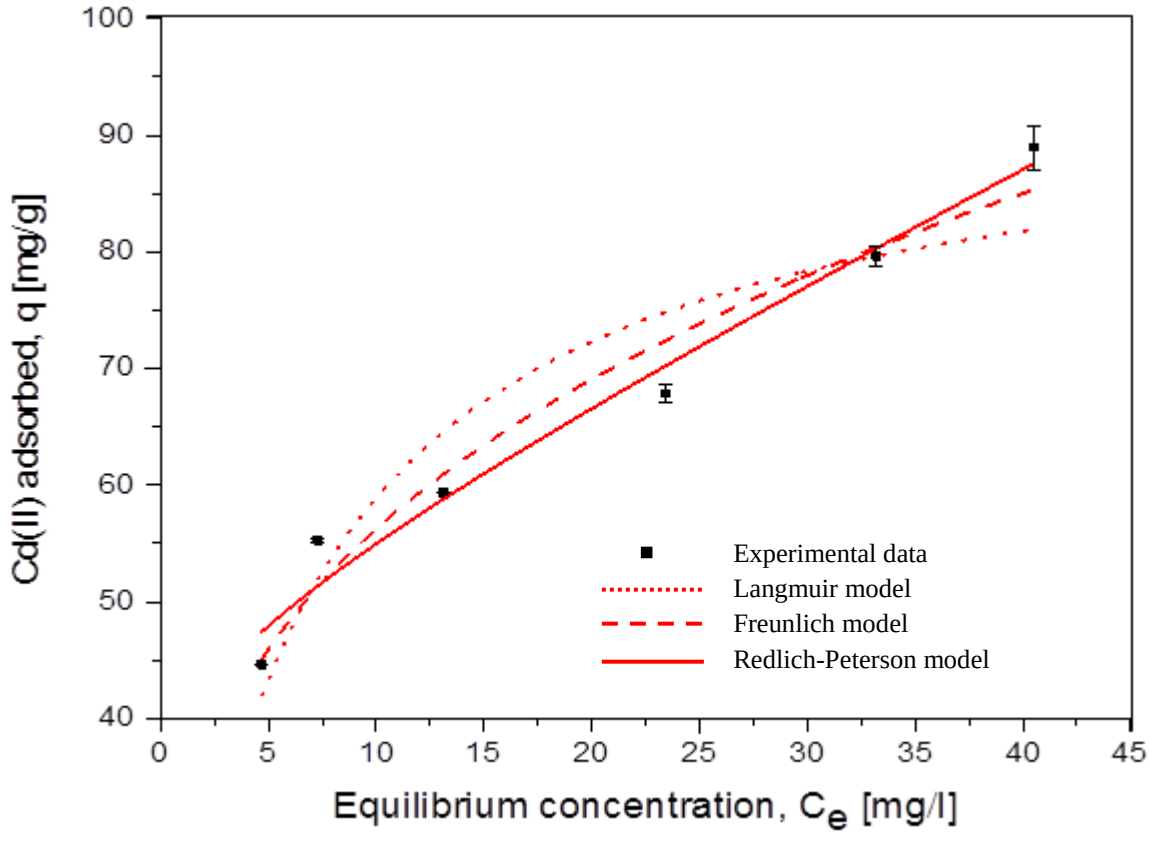


Figure 6.8: Adsorption isotherms with fitted for cadmium (II) adsorption on γ-PGA at room temperature (25°C/298 K). γ-PGA concentration range: 0.1-1.0 g/l; copper concentration: 50 mg/l; equilibrium time: 12 hours (mean ± SD) n=3.

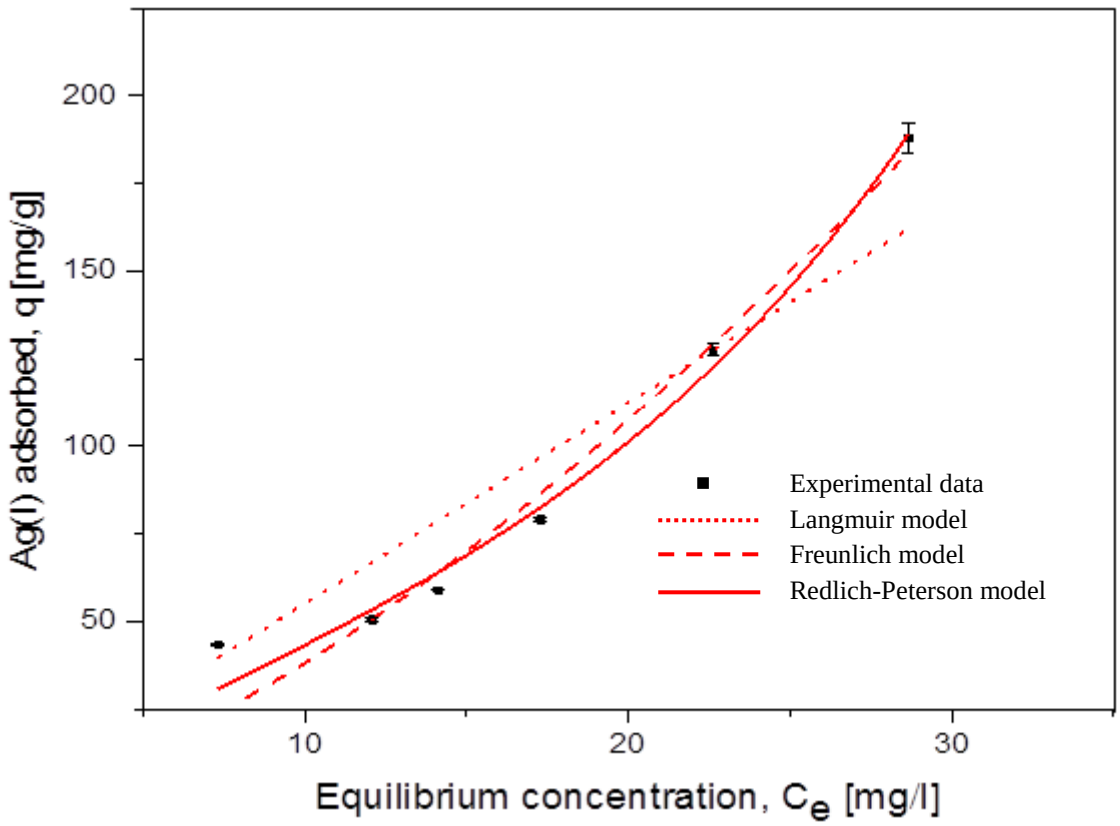


Figure 6.9: Adsorption isotherms with fitted for silver (I) adsorption on γ-PGA at room temperature (25°C/298 K). γ-PGA concentration range: 0.1-1.0 g/l; copper concentration: 50 mg/l; equilibrium time: 12 hours (mean ± SD) n=3.

Table 6.1: Modelled isotherm parameters and error functions for metal ion adsorption on γ-PGA

Metal ion	Langmuir model				Freundlich model				Redlich-Peterson model				
	Qmax (mg/g)	K_L (l/mg)	R^2	X^2	K_F (mg/g)	n_F	R^2	X^2	K_R (l/g)	α_R (l/mg)	β	R^2	X^2
Cu^{2+}	464.32	0.027	0.969	118.15	18.83	1.41	0.987	51.76	5.86	26.177	0.11	0.989	40.76
Zn^{2+}	126.93	0.06	0.851	57.65	16.53	2.15	0.934	26.45	2.81	16.67	0.46	0.987	2.89
Ni^{2+}	277.23	0.029	0.921	128.03	16.35	1.55	0.951	79.67	3.58	36.62	0.147	0.956	71.24
Cd^{2+}	99.62	0.157	0.865	43.13	28.17	3.19	0.949	16.96	0.95	37.95	0.093	0.961	12.576
Ag^{+}	59.83	$8.85x10^6$	0.869	433.94	1.234	0.68	0.962	126.57	0.0603	$9.4*10^5$	3.92	0.976	79.85

6.7 Summary of heavy metals' removal by γ-PGA

The removal of heavy metal ions, which include Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+} and Ag^{+} by HMW γ-PGA obtained from *B*acillus *subtilis* (natto) ATCC 15245 was investigated. The effects of γ-PGA dose, pH, mixture of other heavy metals and molecular weight on metal removal were studied. Metal concentration in heavy metal contaminated water samples before and after γ-PGA treatment was analysed using ICP. The removal performance of γ-PGA was investigated by three isotherm models including Langmuir, Freundlich and Redlich-Peterson isotherms. All experiments were performed in triplicates at room temperature (25^{o}C/298 K) and 150 rpm for 12 hours to attain equilibrium conditions.

Results showed that γ-PGA can adsorb all investigated heavy metal ions. The highest percentage removal of 93.50% was obtained with copper while the lowest was observed in silver. The percentage removal of metal ions increased with increasing γ-PGA concentration. Similarly, pH influenced the removal capacity of γ-PGA. The higher the solution pH, the higher the percentage removal of metals, although the adsorption capacity of γ-PGA in a non-pH controlled system was higher than in a pH controlled system. The mixture of heavy metal ions greatly affected the removal of individual metal ion. The affinities of metal ions for γ-PGA increased in the order $Cu^{2+} > Zn^{2+} > Ni^{2+} > Cd^{2+}$. Removal of heavy metals by γ-PGA was influenced by molecular weight. Adsorption studies revealed that Redlich-Peterson, a three-parameter model that combines the features of both the Langmuir and Freundlich isotherms was found to fit the equilibrium data best compared to Langmuir and Freundlich isotherms, which are two parameters models.

7.0 DISCUSSION

7.1 Precis

This research was carried out to investigate the production of a safe, high yield, water soluble and high molecular weight (HMW) γ-PGA for the removal of heavy metals in wastewaters. To achieve this, five *Bacillus* strains namely: *Bacillus licheniformis* 1525, *Bacillus licheniformis* NCTC 6816, *Bacillus licheniformis* ATCC 9945a, *Bacillus licheniformis* ATCC 9945 (also known as *Bacillus licheniformis* CCRC 12826) and *Bacillus subtilis* (natto) ATCC 15245 (also known as *Bacillus natto* Sawamura) were cultivated in GS medium, medium C and medium E. Amongst the investigated bacteria, *Bacillus licheniformis* ATCC 9945a, *Bacillus licheniformis* ATCC 9945 and *Bacillus subtilis* (natto) ATCC 15245 have been previously studied for the production of γ-PGA (Ko and Gross, 1998, Shih and Van, 2001, Shih *et al*., 2001, Ho *et al*., 2006, Kedia *et al*., 2010 and Bhat *et al*., 2013). However, *Bacillus licheniformis* 1525 and *Bacillus licheniformis* NCTC 6816 have not been reported for the production of γ-PGA, even though *Bacillus licheniformis* NCTC 6816 has been previously extensively studied for subtilisin production (Jacobs *et al*., 1985).

Even though some *Bacillus* species are listed by the Food and Drug Administration as GRAS (generally regarded as safe), the detection of toxins and the genes that produce them in some *Bacillus* strains including those isolated from natural and tap water (Beattie and Williams 1999, Phelps and McKillip 2002 and From *et al*. 2005) has been reported. Therefore, the *Bacillus* strains used in this study were toxigenically analysed to ensure that they do no not produce toxins under production conditions before using them for the production of γ-PGA.

During the course of this study, the production of poly-γ-glutamic acid was accomplished following two major procedures which include: the synthesis of the biopolymer in the fermentation media and the extraction, purification and recovery of dry polymer via lyophilization. Firstly, *Bacillus* strains were cultivated in GS, C and E media following

incubation at 37^{o}C and 50^{o}C (150 rpm) for 96 hours. Three culture media were employed in order to know which culture composition is most suitable for growth and production of a high molecular weight (HMW) and high yield γ-PGA by each strain. GS medium comprises of sucrose as its carbon source while medium E contains glycerol and citric acid as carbon sources. Sucrose had been preferred to other carbon sources for the production of γ-PGA by *B. subtilis* previously (Shi *et al*., 2006, Kedia *et al.*, 2010 and Zeng *et al*., 2013). Similarly, glycerol has been previously reported to enhance γ-PGA production by improving the permeability of cell membrane, thereby enabling the excretion of γ-PGA into the production medium (Du *et al.*, 2005; Wu *et al.*, 2010a and Jeong *et al.*, 2010). Citric acid, an important component of medium E which is known to be the best precursor for γ-PGA production (Du *et al*., 2005) was incorporated into the composition of GS medium to formulate medium C. Thus, medium C and medium E were used to ascertain if the strains used in our study could synthesize γ-PGA in the presence of citric acid precursor while GS medium was on the other hand employed to determine if γ-PGA could be synthesized in the absence of citric acid precursor. Shih and Van (2001) reported that the fermentation temperature and time for γ-PGA production usually ranges from 30 to 37^{o}C and 2 to 5 days respectively. However, Abou-Dobara *et al*. (2011) showed that some *Bacillus* species such as *Bacillus coagulans* and *Bacillus licheniformis* are thermophilic in nature, growing at high temperature up to 70^{o}C and can produce xylanase at an optimum growth temperature of 50^{o}C within 60 hours. Thus, this led to the investigation of the effects of temperature (37^{o}C and 50^{o}C) with time on growth and γ-PGA production by the bacterial strains used in this study.

The second stage of production included: centrifugation to obtain supernatant based polymer, ethanol precipitation, purification of crude polymer via dialysis and recovery of dry polymer via lyophilization. After production, FT-IR and NMR were used to identify and confirm the structure of the biopolymer respectively. The amount of γ-PGA produced by each bacterium

in the different growth media was also evaluated after the fermentation process to determine which medium is best for the growth and production of γ-PGA by each bacterium in terms of yield. The yield of γ-PGA produced differs for each bacterium in each medium. Hence, the characterization of γ-PGA in terms of its form (free acid or salt), molecular weight and crystallinity is important for a good quality product as well as its application. ICP-AES was used to assess the form (free acid or salt) of γ-PGA produced. Aqueous based GPC was used to determine the molecular weight of the polymer while its crystallinity was evaluated using XRD. It was discovered that like the yield, the form, crystallinity and molecular weight of γ-PGA differs for each bacterium in each medium.

After characterization, *Bacillus subtilis* (natto) ATCC 15245 was chosen as the best γ-PGA producer (in terms of yield, form, molecular weight and crystallinity) for further production of γ-PGA used for heavy metal removal application. The γ-PGA produced by *B. subtilis* (natto) ATCC 15245 in medium C has high molecular weight, is non-toxic and completely soluble in water which makes it appropriate for heavy metal removal application since particularly a high molecular weight γ-PGA is necessary for applications in water and wastewater treatment (Bajaj and Singhal, 2011b). The adsorption capability of this HMW γ-PGA was tested on five metal ions (Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, and Ag^{+}). The effects of γ-PGA dose, pH, mixture of other heavy metals and molecular weight on metal removal were studied. ICP-AES was used to analyse metal concentration in heavy metal contaminated water samples before and after γ-PGA treatment. Sorption isotherm models (Langmuir, Freundlich and Redlich-Peterson isotherms) were applied to investigate the removal performance of γ-PGA.

Overall, this study develops the production of HMW γ-PGA from *Bacillus subtilis* (natto) ATCC 15245 and gives insight into the properties (yield, form (acid or salt), molecular

weight and crystallinity) of γ-PGA based on culture conditions and production strains for specific applications. Detailed discussion of this work follows.

7.2 Toxicity test

The toxigenic potential of *Bacillus* strains used in this study were investigated to ensure that they are safe for use following the discovery of toxins and the genes that produce them in some *Bacillus* species (Beattie and Williams 1999 and Phelps and McKillip 2002). Bhat and co-workers had previously analysed the toxigenicity of *Bacillus subtilis* (natto) ATCC 15245 and found that it was toxin free (Bhat *et al*., 2013). Hence, the presence of major enterotoxins and virulence factors in *B. licheniformis* ATCC 9945a and *B. licheniformis* ATCC 9945 were studied and compared to *Bacillus cereus* as a positive control. The results of the toxicity test (**Fig 4.1**) in this study showed that that none of the six toxin producing genes (hbl D/A, nheB, sph, piplc, bceT and entFM) were present in both *Bacillus licheniformis* ATCC 9945a and *Bacillus licheniformis* ATCC 9945. Similarly, physiological analysis (**Fig. 4.2**) indicated that *B. licheniformis* ATCC 9945a and *B. licheniformis* ATCC 9945 showed neither haemolytic nor lecithinase activity. In contrast, four of the toxin producing genes (nheB, entFM, sph, piplc) as well as haemolytic and lecithinase activities were detected in *B. cereus* (the positive control). These results are in agreement with the study of Matarante *et al*. (2004) where toxin producing genes - hbl D/A, nheB, bceT, entFM, sph and piplc were absent in all *Bacillus* strains isolated from food products. Similar results were also found by Bhat *et al*. (2013) who reported the absence of haemolytic and lecithinase activities in *Bacillus subtilis* natto. Consequently, these strains are safe to produce γ-PGA for purification of drinking water and wastewater treatment.

7.3 Growth and production of γ-PGA in different media by *Bacillus* species

The production of γ-PGA was carried out at two different temperatures (37^{o}C and 50^{o}C) to investigate the effect of temperature on the growth and formation of γ-PGA by the *Bacillus* strains investigated in this study.

7.3.1 Growth and production of γ-PGA in different media by *Bacillus* species at 50^{o}C

Growth of *Bacillus* species at the higher temperature of 50^{o}C, as reported by Abou-Dobara *et al*. (2011), was investigated as a means for increasing γ-PGA production efficiency. The results (**Figs. 5.1b to 5.5b**) showed that all *Bacillus* strains initially grew during the first 24 hours before subsequently partially loosing viability in all media. This indicates that the *Bacillus licheniformis* and *Bacillus subtilis* strains used in this study are thermotolerant and can maintain growth at 50^{o}C. A similar result was found by Zeng *et al*. (2013) in which case *Bacillus subtilis* GXA-28 grew and produced γ-PGA at 50°C in liquid fermentation within a short fermentation time. However, the growth of *Bacillu*s strains in our study was not sustained and cell population went into a decline phase. The lack of complete loss of population viability may have been due to sporulation and spore survival.

Extraction of material from the culture of only *Bacillus subtilis* (natto) ATCC 15245 with time showed that γ-PGA was not produced during the 96 hours experimental period, but only glutamic acid was extracted from the medium (**Fig. 5.13**) probably because the L-glutamic acid in the culture medium could not be utilized and converted into γ-PGA by *B. subtilis* (natto) ATCC 15245 at 50°C. Consequently, no further studies at 50°C were performed. In contrast, the strains did grow at 37^{o}C.

7.3.2 Growth and production of γ-PGA in different media by *Bacillus* species at 37°C

The growth and production of γ-PGA at 37°C by all *Bacillus* species used in this study were investigated. Results showed that all investigated strains grew (**Fig. 5.6**) and produced γ-PGA extracellularly in one or all three media (**Fig. 5.7**). These results agree with the investigations of Goto and Kunioka (1992), Du *et al*. (2005), Kedia *et al*. (2010), da Silva *et al*. (2014) and de Cesaro *et al*. (2014) that the optimum fermentation temperature for γ-PGA production by some *Bacillus* species is 37°C. On the other hand, a recent study by Zeng *et al*. (2013) showed that a thermophilic *Bacillus subtilis* GXA-28 could produce γ-PGA at 50°C in liquid fermentation within a short fermentation time. This ability would make γ-PGA production much more cost effective. However, this was not the case in our study, thus, it could be concluded that even though the strains investigated in this study are thermotolerant, none of them are truly thermophilic.

Furthermore, the time courses of γ-PGA production were investigated to determine the effect of incubation time on the production of γ-PGA. It was discovered that γ-PGA was produced only after 72 hours of cultivation (**Table 5.2, 5.8 - 5.12**) with maximum γ-PGA yield obtained after 96 hours of incubation. This is in accordance with the studies of Shih *et al*. (2001) and Berekaa *et al*. (2009) where maximum yields of γ-PGA were obtained at 37°C and after 96 hours of incubation by *Bacillus licheniformis* CCRC 12826 and *Bacillus* sp. strain-R respectively.

It was necessary to study the growth of all investigated strains in different media to determine the influence of medium composition on their growth since previous studies (Du *et al*., 2005 Wu *et al.*, 2010a, Kedia *et al.*, 2010 and Mitsui *et al*., 2011) showed that the composition of culture medium could affect cell growth. This is important because higher cell yields are likely to give higher γ-PGA yields. In addition, the composition of the growth medium may

itself influence γ-PGA synthesis and properties. In order to study the effect of medium composition on cell growth, the investigated strains were grown in three different media (GS, C and E). Results (**Fig. 5.6**) showed that all *B. licheniformis* strains under study (except strain 1525) reached the stationary phase of growth in GS and C media within 24 to 48 hours of growth as against medium E, where all strains took longer time to reach stationary phase. This observation is in accord with the finding of Wu *et al*. (2010a) who found that the presence of high concentration of glycerol in their culture medium initially inhibited cell growth. *Bacillus subtilis* (natto) ATCC 15245 attained a higher cell count when cultivated in GS medium and medium C than in medium E (**Fig. 5.5a**) possibly due to the presence of a vitamin solution in both GS and C media. Similar results were found by Kedia *et al*. (2010). *Bacillus subtilis (natto)* ATCC 15245 is a fastidious strain (Kedia *et al.*, 2010) and thus grew faster in the presence of vitamins.

The formation of γ-PGA in the culture medium makes it viscous which hinders oxygen mass transfer, thereby leading to reduction in oxygen availability. As a result, controlling culture conditions including pH, agitation and aeration is important for optimized γ-PGA production (Richard and Margaritis, 2003, Wu *et al*., 2010b, Bajaj and Singhal, 2011b and Zeng *et al*., 2013). However, batch cultures of all *Bacillus* strains in this study were carried out in shake flask, where there was neither pH nor aeration control, and agitation was only maintained at 150 rpm. This might influence substrate utilization thereby affecting the production of γ-PGA. It was observed that for productions in medium E, the initial medium pH of 7.2 decreased to about 6.5 after 48 hours and later increased to 6.8 at the end of fermentation (**Table 5.1**) while the initial pH of 6.8 decreased to 6.5 after 48 hours until the end of fermentation for productions in GS and C media. It was also noticed that none of the investigated strains in this study reached a cell count higher than 10 log CFU/ml in shake flasks (**Fig. 5.6**). This is possibly due to the lack of pH and/or aeration control in shake flasks

since a previous study performed using fermenter in our laboratory showed that cell counts above 10 log CFU/ml (data not shown) were realized when *B. subtilis* natto was investigated for γ-PGA production. These findings are consistent with those of Cromwick *et al.* (1996) who studied the effects of different pH, aeration and agitation conditions on γ-PGA production by *B. licheniformis* and showed that the optimal yield of γ-PGA (23 g/l) produced by *B. licheniformis* was realised in high aeration conditions (800 rpm and air flow, 2.0 L/min) at pH 6.5 as opposed γ-PGA yield of 6.3 g/l in low aeration conditions (250 rpm and air flow, 0.5 L/min). In other similar studies by Richard and Margaritis (2003) and Thorne *et al.* (1954), maximum biomass and γ-PGA concentration were attained at pH 7.0 by *B. subtilis* IFO3335 and pH 7.4 by *Bacillus subtilis* ATCC 9945 (now *B. licheniformis* ATCC 9945a) respectively. Wu *et al*. (2010b) also investigated the effects of different pH on the production of γ-PGA by *Bacillus subtilis* CGMCC 0833 and found that the optimum pH (6.5) for glutamate consumption was not suitable for cell growth (pH, 7.0) This led them to propose a controlled two phase pH shift approach, where pH value was maintained at 7.0 for 24 hours to achieve optimal cell growth before moving to pH 6.5 for high glutamate utilization.

Therefore, it is definite that pH and aeration control are important for growth and γ-PGA production and optimum pH depends on the bacterial strain. In addition, the scale up of γ-PGA production in a high aerated and pH controlled fermenter would be important for efficient production of γ-PGA.

Following previous reports that *Bacillus* strains produce γ-PGA in the late exponential and stationary phases of growth during fermentation (Buescher and Margaritis, 2007, Mitsui *et al*., 2011 and Bajaj and Singhal, 2011b), the effects of different media on the production and yield of γ-PGA by all *Bacillus* strains used in this study were investigated. It was observed that for all bacterial strains in this study, production media GS and C became viscous after

only 24 hours until the end of cultivation while viscosity became evident in medium E after 48 hours indicating the formation of γ-PGA and was linked to the stationary phases of growth for these media. This is in agreement with the report of Bajaj and Singhal (2011b) that γ-PGA production is known to occur towards the end of the logarithmic and during the stationary phases of growth. This is also similar to the studies of Ito *et al*. (1996) and Mitsui *et al*. (2011). Ito *et al*. (1996) found that the production medium of γ-PGA became highly viscous due to the accumulation of polymer along with bacterial growth when *B. subtilis* TAM-4 was investigated for γ-PGA production. Likewise, Mitsui *et al*. (2011) showed that the production of γ-PGA started at the early stationary phase of growth and continued up until the end of fermentation. The *pgsB* gene encoding the γ-PGA synthase complex (pgsBCA) which is considered to have a catalytic site for polymerization of γ-PGA and is dependent on ATP (Ashiuchi *et al*., 1999a, Urushibata *et al*., 2002a and Candela and Fouet, 2006) was expressed in the early stationary phase of growth in *Bacillus subtilis* (natto) according to Kimura *et al*. (2009). This indicates why γ-PGA is produced in the stationary phase of growth.

γ-PGA was extracted by the method described in **3.1.7** and the recovered γ-PGA in form of dry powder was weighed to determine its yield. The yields of γ-PGA produced by different *Bacillus* strains in GS, C and E media are presented in **Fig. 5.7**. *Bacillus licheniformis* 1525 and *Bacillus licheniformis* NCTC 6816 produced relatively low γ-PGA compared to the other strains investigated in this study. It could be that strains 1525 and NCTC 6816 generally have low metabolic synthesizing capability since other strains under the same condition had better yields and may require specific nutritional needs for optimal γ-PGA production. Since these two strains have not been previously investigated for the production of γ-PGA (except for a previous study carried out in our laboratory), there is no information on the culture conditions and requirements of these strains. This study has however ascertained the production capability of γ-PGA by *B. licheniformis* 1525 and NCTC 6816. The influence of growth

parameters and culture conditions on the production of γ-PGA by *B. licheniformis* 1525 and NCTC 6816 would need to be investigated in order to confirm whether they are capable of producing higher yields of γ-PGA.

The yields of γ-PGA produced by *Bacillus licheniformis* ATCC 9945a in GS medium, medium C and medium E were comparable ($P > 0.9999$) even though the yields of γ-PGA from GS medium and medium C were slightly higher than in medium E (**Fig. 5.7**). This could indicate that like sucrose (GS and C media), glycerol (medium E) is also a good carbon source for the production of γ-PGA. The slightly higher yields of γ-PGA in media containing sucrose are consistent with the investigations of Shi *et al*. (2006) and Zeng *et al.* (2013) where sucrose has been chosen over other carbon sources for the production of γ-PGA using *B. subtilis* previously. However, the mechanism by which sucrose enhances the production of γ-PGA production is unknown. This result is also in agreement with those of Birrer *et al*. (1994) and Cromwick and Gross (1995a) where γ-PGA was produced by *Bacillus licheniformis* ATCC 9945a in medium E.

There was no production of γ-PGA when *Bacillus licheniformis* ATCC 9945 (*Bacillus licheniformis* CCRC 12826) was cultured in GS medium and medium C **(Fig. 5.7)** despite initial cell growth in both media (**Fig. 5.4**). This could possibly be because *Bacillus licheniformis* ATCC 9945 was unable to utilize the nutrients contained in GS and C media for γ-PGA production. This result is in accordance with the findings of Shih *et al*., (2001) and Shih *et al*. (2002) who had previously investigated the effects of different nitrogen and carbon sources on the production of γ-PGA by *Bacillus licheniformis* CCRC 12826 and found that γ-PGA was not produced when lactose, glucose or fructose was employed as a carbon source in the production medium but that glycerol, glutamic acid and citric acid are required for growth and production of γ-PGA by *B. licheniformis* CCRC 12826. They also

showed that ammonium chloride was the best nitrogen source for the cultivation of *B. licheniformis* CCRC 12826 in the production medium (medium E). Ammonium ions are necessary for the endogenous production of glutamic acid from citric acid (Kunioka and Goto, 1994).

Results (**Fig.5.7**) showed that *Bacillus subtilis* (natto) ATCC 15245 produced significantly higher γ-PGA in GS and C media than in medium E ($P < 0.0001$). The reason for this could be that, *B. subtilis* (natto) ATCC 15245, which is known to be a fastidious organism in nature and require specific nutrients for growth (Kedia *et al*., 2010) grew faster in vitamin supplemented media (GS and C) than in medium E (**Fig. 5.5**) and produced higher concentrations of γ-PGA. This result is similar to that obtained by Kedia *et al*. (2010) who reported the production of high yield γ-PGA by *Bacillus subtilis* (natto) ATCC 15245 in GS medium. Results (**Fig.5.7**) also showed that even though higher yields of γ-PGAs were obtained in vitamin supplemented media than in medium E, γ-PGA can be produced by *B. subtilis* (natto) ATCC 15245 in the absence of vitamins and does not necessarily require vitamins for growth and production of γ-PGA. This is in accordance with the study of Ito *et al*. (1996), which showed that *B. subtilis* TAM-4, a glutamic acid independent (unlike other γ-PGA producing *B. subtilis* (natto) strains) does not require the vitamin biotin for growth and γ-PGA production. Another study by Mitsui *et al*. (2011) showed that γ-PGA production by *B. subtilis* (natto) strain NAFM5 was poor in LB medium (rich in nutrients), but that γ-PGA production was enhanced following the addition of sodium glutamate and sucrose to the medium. The nutritional requirements of bacteria for the production of γ-PGA varies, for example, some γ-PGA producers like *Bacillus subtilis* IF03335 and *B. subtilis* (chungkookjang) require vitamin for γ-PGA production (Goto and Kunioka, 1992 and Ashiuchi *et al*., 2001). It can therefore be assumed that the addition of vitamins might

enhance the production of γ-PGA by *B. subtilis* species but that it is not compulsory for its production.

Overall, it was discovered that all the investigated *Bacillus* strains could produce γ-PGA in the presence (GS and C media) and absence (medium E) of vitamins as well as with (C and E media) and without (GS medium) citric acid precursor. This investigation shows that the yield of γ-PGA is not only dependent on the bacterial strain used for production, but also on the media composition and culture conditions for optimal utilization and conversion of nutrient to γ-PGA. This indicates that the metabolic pathways for γ-PGA production might vary for different bacterial strains.

7.4 Identification and Characterization of biopolymer

The materials produced by all *Bacillus* strains used in this study from GS, C and E media at 37°C after 96 hours were analysed using FT-IR spectroscopy. Each investigated *Bacillus* strain produced γ-PGA in one or all three media, as identified by FT-IR spectroscopy (**Figs 5.8 – 5.12**). The FT-IR spectra of γ-PGA samples produced by all strains showed high similarity to that of a commercially available γ-PGA sample. The FT-IR spectra of all γ-PGA samples including that of the commercial sample showed characteristic strong hydroxyl absorption at about 3350-3450 cm^{-1}, carbonyl absorption at about 1395-1410 and C-N groups' absorption peaks from 1070 cm^{-1} to 1138 cm^{-1}. The strong absorption peaks observed at approximately 620 cm^{-1} are characteristic of and N-H oop bending. The absorption peak at about 1738 cm^{-1} show distinctive C=O stretch while the peaks ranging from 1575 cm^{-1} to 1619 cm^{-1} are characteristics of amide groups. These results are consistent with those obtained by Ho *et al*. (2006) who had studied the structural characteristics of γ-PGA and its salts using FT-IR. The presence of these absorption peaks at specific wavelengths in the FT-

IR spectra of γ-PGAs produced in this study confirms the presence of hydroxyl, carbonyl, amide and carboxyl groups as characterized by Ho *et al* (2006).

Solubility is an important property of γ-PGA since its application largely depends on it. For instance, the ability of insoluble γ-PGA to sequester water molecules (absorbability) makes it suitable for use as hydrogel (Ho *et al*., 2008 and Tsao *et al*., 2011). The protonated (acid) form of γ-PGA is insoluble in water but is soluble in organic solvents such as DMSO (Kubota *et al*., 1992 and Ho *et al*., 2006) whereas, the ionized (salt) forms are soluble in water. According to Tolentino (2014), γ-PGA is a polyelectrolyte and its ionization state to a large extent determines its solubility. Na^+ γ-PGA has an acid dissociation constant (pK_{α}) of 4.09 (Ho *et al*., 2006), hence its form as well as its conformation depends on the pH of the solution. At pH 2, γ-PGA is un-ionized and therefore, its acid form is predominant and adopts α-helical conformation (Ho *et al*., 2006 and Tolentino 2014). As the degree of ionization increases with increasing pH, hydrogen bonding disintegrates, turning the carboxyl groups into anionic groups. Approximately 50% of α-carboxyl groups ionize into α-anionic groups at pH 4.09. When hydrogen bonding is broken and α-carboxyl group is ionized, γ-PGA changes from insoluble α-helix into the soluble linear random-coil conformation until pH reaches about 6.0. At pH $\geq$ 6 all hydrogen bonding vanishes and insoluble α-helical γ-PGA completely changes into soluble linear random-coil conformation turning all carboxyl groups into pendant anionic groups that are free for binding of molecules (Ho *et al*., 2006 and Ye *et al*., 2006).

Hence, high yield γ-PGA samples produced by *B. licheniformis* ATCC 9945a, *B. licheniformis* ATCC 9945 and *B. subtilis* (natto) ATCC 15245 form GS, C and E media in our study were analysed using ICP-AES to determine their forms and to know if the bacterium and/or medium used in the production of γ-PGA influenced its form. The results

(**Table 5.3**) showed that γ-PGA samples produced by these three strains were a mixture of both the acid and Na^+ form of γ-PGA. The γ-PGAs produced from medium E had more Na^+ form than those from GS and C media. Similar results were obtained by Thorne *et al*. (1954) who isolated a sodium salt of γ-PGA when *B. licheniformis* ATCC 9945a was cultured in medium E. All high yield γ-PGA samples (except for γ-PGA produced by *B. licheniformis* ATCC 9945a in medium E which contained minor dust-like insoluble particles) were completely soluble in water which suggests that these γ-PGA(s) are water soluble and random coiled. This finding agrees with the study of Thorne *et al*. (1954) and Ho *et al*. (2006).

Furthermore, the crystallinity of γ-PGA can also influence its solubility in water. The report of Wainwright *et al*. (1982) on XRD studies of polymeric substances has shown that polymers can be amorphous or crystalline. According to their study, for a polymer to be crystalline, it has to be linear and the crystallization of a linear polymer requires orderliness in its chain arrangement indicating that regions where helically coiled polymers are closely packed in a regular manner are referred to as crystalline regions while those of randomly arranged flexible polymers are amorphous (Wainwright *et al*., 1982 and Schultz, 2013). When the degree of crystallinity in a material is less than 20%, crystalline regions behave like crosslinks within an amorphous polymer whereas, when the degree of crystallinity is over 40%, crystalline regions tend to become so closely packed together, and polymer turns out to be quite stiff (Wainwright *et al*., 1982). Therefore, the crystallinity of high yield γ-PGA(s) produced in this study was analysed to confirm the relationship between crystallinity and solubility using XRD. The results (**Fig. 5.14**) showed that with the exception of γ-PGA produced by *B. licheniformis* ATCC 9945a in medium E (**Fig. 5.14d**) is semi-crystalline (showing some degree of crystallinity at 22, 23, 26, 32, 33 and 37 degrees of 2θ) while all other high yield γ-PGAs produced in this study are amorphous. The amorphous γ-PGA which

is soluble in water has potential application as an adsorbent for metal ion removal whereas the insoluble semi-crystalline γ-PGA does.

Molecular weight is another important property of γ-PGA. According to Bhat (2012), the molecular weight of γ-PGA can be influenced by: how many glutamic acid residues bacteria are able to polymerize before transporting it outside the cell, the presence of degradation enzymes and composition of the growth medium. The medium used to produce γ-PGA by various bacteria is significant since it directly affects the properties of γ-PGA. For instance, according to Sung *et al*. (2005b), in *B. subtilis (chungkookjang*) altering the NaCl concentration in the medium between a particular range say 0 and 100 g/L could regulate the molecular weight of γ-PGA. Likewise, the molecular weight of γ-PGA also differs based on the strains used. For example *Bacillus sp.* RKY3 and *B. subtilis* (chungkookjang) produces γ-PGA of 10-50 k Da and in excess of 10,000 k Da respectively even under similar cultural conditions (Buescher and Margaritis, 2007). Molecular weight is of importance when optimizing γ-PGA for specific application as different applications usually require γ-PGA of different molecular weights (Ashiuchi and Misono, 2002). For instance, low molecular weight γ-PGAs of $< 20 \times 10^3$ Da and 14×10^3 Da showed high antifreeze activity in the studies of Mitsuiki *et al.* (1998) and Shih *et al.* (2003) respectively whereas, a higher molecular weight γ-PGA (9.9×10^5 Da) was shown to be effective for wastewater treatment applications (Inbaraj *et al.*, 2006). High molecular weight γ-PGA is very promising for applications such as heavy metal adsorption and flocculation of organic and inorganic substances in water and wastewater treatment. According to Abe *et al*. (1997), an extracellular enzyme, PGA depolymerase produced alongside γ-PGA during production by *Bacillus subtilis* TAM-4 degraded the γ-PGA produced and as a result, the molecular weights of γ-PGAs may vary depending on the activity of PGA depolymerase. Mitsui *et al*. (2011) also investigated the effects of cell wall lytic enzymes (LytE, LytF, CwlO and CwlS) on the

molecular weight of γ-PGA by *Bacillus subtilis* (natto) and found that CwlO was responsible for γ-PGA degradation. Production of high molecular weight γ-PGA from *Bacillus subtilis* (natto) has proved to be challenging for the following reasons: γ-PGA synthesis or elongation is sometimes coupled with degradation of γ-PGA towards the later stages of fermentation; and the PGA synthetase complex itself is not stable. Nevertheless, Park *et al*., (2005) found that the cultivation of *B. subtilis* subsp. *chungkookjang* in a medium with high concentration of ammonium sulphate yielded super-high-molecular-weight γ-PGA ($\geq 2 \times 10^6$ Da) without the aforementioned problems.

Hence, the effects of *Bacillus* strains and culture composition on the molecular weight of high yield γ-PGA(s) produced in this study were analysed using GPC. Results (**Table 5.4**) showed that the molecular weight of γ-PGA(s) produced in our study depended on the media used. γ-PGA(s) produced from medium C had the highest molecular weights of ~$1.6 * 10^6$ Da. It was discovered that the concentration of NaCl in the production medium influenced the molecular weight of γ-PGA since *B. licheniformis* ATCC 9945a and *B. subtilis* (natto) ATCC 15245 produced γ-PGAs of 1.42×10^6 Da and 1.47×10^6 Da respectively in GS medium containing 0.5% NaCl compared to ~2.57×10^5 Da produced by *B. subtilis* (natto) ATCC 15245 in GS medium of 5% NaCl. This finding is consistent with the investigations of Ashiuchi *et al*. (2001a) and Wei *et al*. (2010) that γ-PGAs with lower molecular weights were produced in culture media containing high NaCl concentrations compared to low NaCl concentrations. Ashiuchi *et al*. (2001a) indicated that PGA depolymerase might be halophilic in their strain, and can only degrade γ-PGA effectively in the presence of high NaCl concentration. The molecular weights of γ-PGAs (**Table 5.4**) produced in our study also showed that the addition of critic acid to the GS culture medium containing 0.5% NaCl (medium C) further increased the molecular weights of γ-PGAs produced by *B. licheniformis* ATCC 9945a and *B. subtilis* (natto) ATCC 15245. The activity of degradation enzymes is

possibly lower in the presence of both citric acid and low NaCl concentration than in the later alone. The combined effect of citric acid and low concentration of NaCl on the molecular weight of γ-PGA has not been previously investigated; thus, this study has recognized a relationship between citric acid + low concentration of NaCl and molecular weight of γ-PGA. The influence of medium composition on the activity of degradation enzymes in γ-PGA production by *B. licheniformis* ATCC 9945a and *B. subtilis* (natto) ATCC 15245 would need to be investigated in order to confirm the role of citric acid + low concentration of NaCl in the inactivation of these enzymes and/or elongation of γ-PGA. In addition, the molecular weight of γ-PGAs produced in our study was dependent on the *Bacillus* strain used (**Table 5.4 & 5.5**) as the molecular weights of γ-PGAs produced by *B. subtilis* (natto) ATCC 15245 was consistently higher than those produced by *B. licheniformis* ATCC 9945a in all media. The highest molecular weight γ-PGA of 1.65×10^6 Da in this study was obtained by *B. subtilis* (natto) ATCC 15245 from medium C and used for further studies.

After characterization of the high yield γ-PGAs produced in our study, γ-PGA produced by *B. subtilis* (natto) ATCC 15245 in medium C was chosen for heavy metal removal application based on the following reasons: *B. subtilis* (natto) ATCC 15245 is a non-toxic source of γ-PGA, produced the highest yields of γ-PGA in both GS medium (11.69 g/l) and medium C (11.59 g/l) and secreted the highest molecular weight (1.65×10^6 Da) γ-PGA in medium C. After selecting the γ-PGA produced by *B. subtilis* (natto) ATCC 15245 from medium C, it was further analysed by proton NMR in order to confirm the structure and the form of this γ-PGA. The result (**Fig. 5.15**) showed that the ^{1}H-NMR spectrum for γ-PGA produced by *B. subtilis* (natto) ATCC 15245 from medium C displays all peaks to that from the commercially available γ-PGA. ^{1}H-NMR for this γ-PGA shows chemical shifts at 3.98 ppm for α-CH proton, 1.98 ppm & 1.80 ppm for β-CH_2 proton and 2.19 ppm for γ-CH_2 proton. This result is in accordance with those reported by Ho *et al*. (2006) and Kedia *et al*. (2010). The presence

of these peaks at specific chemical shifts in the ^{1}H-NMR spectrum of γ-PGA produced by *B. subtilis* (natto) ATCC 15245 from medium C in this study confirms that this γ-PGA is sodium γ-PGA (Na^{+} γ-PGA) as characterized by Ho *et al* (2006).

Na^{+} γ-PGA is also tasteless (Ho *et al*., 2006) and hence can be used in wastewater treatment and for purification of drinking water.

7.5 Removal of heavy metals by poly-gamma-glutamic acid (γ-PGA)

γ-PGA is an anionic polymer which comprises of repeating D- and/or L-glutamic acid units polymerised through amide linkages between α-amino acid and γ-carboxylic acid groups (**see Fig.1**) with molecular weights ranging from 10×10^{3} to about 2×10^{6} Da (Shih and Van, 2001 and Ho *et al*., 2006). High molecular weight γ-PGA has benefits over low molecular weight γ-PGA such as more charge densities and higher viscosities at lower concentrations (Prescott and Louis, 2008). Since the repeating glutamic acid units in γ-PGA are connected via γ-peptide linkage leaving the α-carboxyl groups free for binding to different compounds (Inbaraj and Chen, 2012), a high molecular weight γ-PGA (which invariably means a long polymer) will have more free functional groups to act as binding sites for positively charged metals compared to low molecular weight γ-PGA.

Although γ-PGA has been previously investigated for its ability to remove heavy metals from aqueous solution (Yao *et al*., 2007, Inbaraj *et al*., 2009, Hadju *et al*., 2012, Bodnár *et al*., 2013 and Chang *et al*., 2013), there is no report on whether the molecular weight of γ-PGA affects the removal of metals. This study thus investigated the effect of molecular weight of γ-PGA on its heavy metal removal capability. Additionally, the effects of pH, γ-PGA concentration and other competing metal ions on the removal of Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, and Ag^{+} by γ-PGA were assessed.

7.5.1 Effect of γ-PGA concentration on metal ion adsorption

The dosage of adsorbent plays an important role in the process of adsorption since the more available active binding sites there are for adsorption, the higher the percentage removal of the sorbate (El-Sayed and El-Sayed, 2014). The effect of γ-PGA concentration on removal of Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, and Ag^{+} was therefore investigated by varying the concentration of γ-PGA from 0.1 to 1.0 g/l at room temperature in a non-pH controlled system. Results (**Fig. 6.1**) showed that percentage removal of all investigated metal ions increased with increasing γ-PGA concentration due to the presence of more binding sites for metal ions at high increasing γ-PGA concentrations. The highest metal removal of 93.50% by γ-PGA was obtained for Cu^{2+}. Similar results were obtained by Inbaraj *et al*. (2006) who found that the percentage removal of basic dyes increased with high γ-PGA doses. Meena *et al*. (2005) also found that the removal of heavy metals increased rapidly with increase in the dosage of the adsorbent due to more available exchange sites.

7.5.2 Effect of pH on metal ion adsorption

The pH of the solution is also important when determining metal adsorption since the mechanism of adsorption involves the binding of solutes by sorbents (Inbaraj *et al*., 2009). Furthermore, studying the influence of pH on the adsorption of heavy metals by γ-PGA which is a polymer with several charged surface functional groups will be of great significance as pH has the ability to affect the dissociation of cation exchange groups and conformation of γ-PGA as well as stability of the metal complexes formed with γ-PGA (Inbaraj *et al*., 2009). Thus the effect of pH on the removal of Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+} and Ag^{+} by γ-PGA was investigated. Results presented in **Fig. 6.2** showed that no adsorption took place in all metal ions until pH values reached 3 and the adsorption capacity of γ-PGA for heavy metal ions depended on the pH of the solution. The amount of metal ions adsorbed

increased with rising pH values until the point where metal ions started to precipitate which was detected at pH 6.0, 7.0, 7.5, 8.0 and 7.5 for Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+} and Ag^{+} respectively. These results are in agreement with those obtained by Yao *et al*. (2007), Inbaraj *et al*. (2009) and Hadju *et al*. (2012). According to Siao *et al*. (2009), for adsorbents that are rich in carboxyl groups, the binding of cation greatly depends on the pH of solution and pK_a of the material. The pK_a of γ-PGA is 4.09 (Ho *et al*., 2006), so at pH less than 4.09, the α-carboxyl groups of γ-PGA are also in the un-ionized form thereby preventing the adsorption of metal ions (Siao *et al*., 2009 and Inbaraj *et al*., 2009). In addition, because γ-PGA is un-ionized at low pH, it possesses a stable α-helix conformation due to strong intra-molecular hydrogen bonds (Ho *et al*. (2006) which may hinder the metal ions access to the functional groups (Yao *et al*., 2006, Siao *et al*., 2009 and Inbaraj *et al*., 2009). However, at higher pH values, γ-PGA becomes deprotonated and the α-COOH groups are ionized into anionic α-COO^- groups (Ho *et al*., 2006), making more functional groups of γ-PGA available for the metals to bind (Yao *et al*., 2007 and Siao *et al*., 2009).

It was interesting to know that even though the amount of metal ion adsorbed increased with increasing pH values before metal ions started to precipitate, the adsorption capacity of γ-PGA in a non-pH controlled system (**Fig. 6.1**) was higher than in a pH controlled system (**Fig.6.2**) in our study. This outcome may be due to fact that since the γ-PGA used in this study is already in ionized form (water soluble linear random-coiled γ-PGA), adjusting the pH using NaOH might have interfered with the complexation of metal ions-γ-PGA. Alternatively, the sorption mechanism involved in the removal of metal ions by γ-PGA might have been complexation and/or chelation since the sorption of metal ions on natural sorbents is a complex process that may include ion exchange, complexation, chelation and micro-precipitation amongst others and different sorption process may possibly cause different changes in the pH (Gyliene *et al*., 2002). For ion exchange and micro-precipitation, pH

decreases following sorption of metal ions whereas, complexation and chelation may happen without pH adjustment (Gyliene *et al*., 2002). However, further investigation would need to be carried out in order to confirm this possibility.

7.5.3 Effect of mixed heavy metal ions on individual metal ion adsorption

Heavy metals are usually present in the environment in mixtures (Tsiridis *et al*., 2006). It is therefore important to investigate the effect of mixture of different heavy metal ions on individual metal ion in order to assess the selectivity of heavy metal adsorption. Moreover, it is essential in determining the possibility of using an adsorbent for the recovery of a particular target metal ion (Inbaraj *et al*., 2009). The effect of the mixture of all divalent heavy metal ions under study (Cu^{2+} + Zn^{2+} + Ni^{2+} + Cd^{2+}) on the adsorption of each of the metal ions by γ-PGA was assessed. The results obtained (**Fig. 6.3**) showed a decrease in the amount of each metal ion adsorbed compared to results obtained in a single metal ion adsorption process in this study (**Fig. 6.1**) indicating competition amongst these heavy metal ions. Similar results were found by Inbaraj *et al*. (2009) who showed that the presence of other metal ions could hinder the adsorption of individual metal ion by γ-PGA. The affinities of heavy metal ions for γ-PGA followed the order: Cu^{2+} > Zn^{2+} > Ni^{2+} > Cd^{2+} showing a better selectivity of γ-PGA for copper followed by zinc, nickel and then cadmium (**Fig. 6.3**). This order conforms to that of metal hydrolysis constants (Kobayashi *et al*., 1998 and Gyliene *et al*., 2002). Metal ions whose hydroxides are less soluble were more easily sorbed. Similar results were found by Bernal *et al*. (2000), Gyliene *et al*. (2002) and Yao *et al*. (2007) when silica gel-immobilized 8-hydroxyquinoline, chitin and γ-PGA were used for heavy metal adsorption respectively.

7.5.4 Effect of molecular weight on metal ion adsorption

As earlier mentioned (**see section 7.4**), the molecular weight of γ-PGA is an important characteristic on which its application depends and γ-PGAs of different molecular weights are required for different applications. The effect of molecular weight of γ-PGA as related to its chain length on the removal of heavy metal ion was studied by varying the concentrations of both low molecular weight ($2.56 * 10^5$ Da) and high molecular weight ($1.65 * 10^6$ Da) γ-PGAs (0.1 to 1.0 g/l) for the adsorption of Cu^{2+}. Cu^{2+} was selected for investigation since the adsorption capacity of γ-PGA was highest for Cu^{2+} amongst the investigated metal ions. The results obtained (**Fig. 6.4**) showed that the molecular weight of γ-PGA strongly affects its adsorption capacity. The maximum amount (93.50%) of Cu^{2+} sorbed by HMW γ-PGA was higher than that (59.48%) sorbed by LMW γ-PGA. This outcome is in agreement with that obtained by Shih *et al*. (2001) who in their study found that the molecular weight of γ-PGA influences their flocculating activity. According to Michaels (1954) and Gutcho (1977), high molecular weight polymers which are typically long and have adequate numbers of free functional groups can bridge between various suspended particles of opposite charges. This could explain why HMW γ-PGA binds heavy metals.

7.6 Adsorption isotherm

Adsorption isotherms, which define the relationship between the mass (q) of the solute (heavy metal ion) adsorbed per unit mass of adsorbent (γ-PGA) and the solute concentration in the solution at equilibrium (C_e) are fundamental criteria required for describing an adsorption system (Foo and Hameed, 2010). The equilibrium data are important in optimizing the design variables for any adsorption process (Inbaraj *et al*., 2006).

Adsorptions of Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+} and Ag^{+} by γ-PGA at fixed initial heavy metals concentration (50 mg/l) and room temperature (25^oC/298 K) were studied by varying γ-PGA

concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 g/l) in this research. Adsorption isotherms were modelled with three equilibrium models including Langmuir, Freundlich and Redlich-Peterson isotherms (**Table 3.3**) to assess which model best fits the experimental data. Langmuir and Freundlich isotherms models are two parameter isotherms while and Redlich-Peterson isotherm model is a three parameter isotherm. Langmuir isotherm which assumes monolayer adsorption describes homogeneous adsorption in which all sites possess equal affinity for the adsorbate with no transmigration of the adsorbate in the plane of the surface (Foo and Hameed, 2010). Freundlich isotherm on the other hand is not limited to the formation of monolayer and describes the non-ideal and reversible adsorption on heterogeneous surfaces. This empirical equation model can be used for multilayer adsorption with non-uniform distribution of adsorption energy over the heterogeneous surfaces (Foo and Hameed, 2010). Redlich-Peterson isotherm is a hybrid isotherm which incorporates three parameters into an empirical equation by incorporating the features of both Langmuir and Freundlich isotherms. This model can be applied to either homogeneous or heterogeneous systems due to its flexibility and approaches Freundlich isotherm model at high concentration when the exponent β is close to zero but agrees with the Langmuir isotherm at low concentration as β values are close to one (Foo and Hameed, 2010). Results (**Figs. 6.5 – 6.8 & section 6.6**) showed that an increase in the concentration of γ-PGA lead to increased adsorptions for all metal ions. It was also found that Redlich-Peterson isotherm model best fitted the experimental data at room temperature (298 K) as demonstrated by high R^2 and low X^2 values (**Table 6.1**) obtained compared to those obtained with Langmuir and Freundlich isotherms models. Amongst the autonomous two-parameter models, Freundlich isotherm better described the equilibrium data than the Langmuir model due to its higher R^2 and lower X^2 values (**Table 6.1**). Moreover, the isotherm approaches a Freundlich type of adsorption since the values of Redlich-Peterson exponent β are close to zero (**Table 6.1)** for all metals,

indicating a heterogeneous nature of adsorption for all metal ions investigated. The high Qmax value of 464.32 mg/g derived from the Langmuir model for Cu^{2+} corresponds to the highest metal removal of 93.50% for Cu^{2+}. These results are similar to those obtained by Inbaraj *et al*. (2009) who reported that the Redlich-Peterson isotherm best described their equilibrium data and that their isotherms tended towards a Freundlich-type adsorption.

Overall, since HMW γ-PGA performed well in removing heavy metals from water samples containing metals, it can be adopted as a biosorbent for removing heavy metals in water and wastewater treatment application. In addition, this biopolymer could offer green a solution to water and waste water treatment plants as well as in various industries utilizing heavy metals due to its non-toxic and biodegradable nature.

8.0 CONCLUSION

Poly-γ-glutamic acid (γ-PGA) is a biopolymer made up of repeating units of L-glutamic acid, D-glutamic acid or both. γ-PGA is water soluble, non-toxic, biodegradable and non-immunogenic, and can therefore be used safely in a variety of applications that are increasing rapidly. It is important to study the production of γ-PGA by different bacteria and to characterize the γ-PGAs produced by these bacteria, as this would provide insights for development of high quality polymers suitable for use in different applications.

In this study, the production of a safe, high yield, water soluble and high molecular weight (HMW) γ-PGA by five different bacteria was investigated in three media (GS, C and E) for the removal of heavy metals in wastewaters. Three of these bacteria (*Bacillus licheniformis* ATCC 9945a, *Bacillus licheniformis* ATCC 9945 and *Bacillus subtilis* (natto) ATCC 15245) have been previously reported for the production of γ-PGA whereas the other two (*Bacillus licheniformis* 1525 and *Bacillus licheniformis* NCTC 6816) have not been reported for γ-PGA production.

In general, the *Bacillus* strains used in this study are safe and can be used to produce γ-PGA for purification of drinking water and wastewater treatment. It was found that 37^{o}C and 96 hours are the ideal temperature and incubation time for growth and production of γ-PGA by all strains investigated in this study. Medium composition was found to influence the growth of bacterial strains as strains cultured in GS and C media reached the stationary phase earlier that those cultured in medium E. In addition, it was seen that pH and aeration control are important for γ-PGA production and that optimum pH depends on the bacterial strain.

The highest γ-PGA yields of 11.69 g/l and 11.59 g/l were produced by *Bacillus subtilis* (natto) ATCC 15245 in GS medium and medium C respectively. All bacterial strains (except *Bacillus licheniformis* ATCC 9945) investigated in this study could produce in the presence (GS and C media) and absence (medium E) of vitamins as well as with (C and E media) and

without (GS medium) citric acid precursor. It was discovered that the production of γ-PGA is not only dependent on the bacterial strain used for production, but also on the media composition and culture conditions for optimal utilization and conversion of nutrient to γ-PGA.

The properties of the γ-PGAs produced are important since the application of γ-PGA largely depends on its properties. All bacteria produced γ-PGA in one or all three media as established by FT-IR spectroscopy. It was found that the solubility of γ-PGAs produced in this study was determined by its crystallinity as the amorphous γ-PGAs were completely soluble in water which is very important in wastewater treatment whereas semi-crystalline γ-PGA was not soluble in water.

The molecular weight of γ-PGA is particularly important as it determines the application for which it can be used. A low molecular weight γ-PGA is mainly required for medical applications whereas a high molecular weight γ-PGA can be used for applications in the water and wastewater treatment industry. Five bacteria cultured in three different media produced γ-PGAs with molecular weights ranging from 2.56×10^5 Da to 1.65×10^6. The molecular weights of γ-PGAs produced in this study were dependent on both bacteria and medium used for their production. The molecular weight of γ-PGA was strongly influenced by the presence of either low (0.5%) NaCl concentration or citric acid + low (0.5%) NaCl concentration in the culture medium, with the latter having a greater effect. This is the first report to recognize a relationship between citric acid + low concentration of NaCl and molecular weight of γ-PGA.

After the characterization of high yield γ-PGAs produced by three *Bacillus* strains in all media, the best γ-PGA producer was selected for further studies. The γ-PGA produced by *Bacillus subtilis (natto)* ATCC 15245 in medium C was chosen for heavy metals removal

application for the following reasons: *B. subtilis* (natto) ATCC 15245 is a non-toxic source of γ-PGA, produced a high γ-PGA yield of 11.59 g/l in medium C and secreted the highest molecular weight (1.65×10^6 Da) γ-PGA in medium C. ^{1}H-NMR further confirms this γ-PGA to be Na^+ γ-PGA which is tasteless (Ho *et al*., 2006), an essential attribute for water treatment.

Adsorbents are often employed in the removal of heavy metals from metal contaminated water. However, biopolymers such as γ-PGA present an eco-friendly approach to metal removal due to its non-toxicity, biodegradability and solubility. Although the metal removal capability of γ-PGA has been previously investigated, the effect of molecular weight of γ-PGA on metal removal has not been studied. It was found that the removal of metals by γ-PGA was more dependent on the concentration of γ-PGA than the solution pH. The presence of interfering metal ions could hinder the adsorption of individual metal ion by γ-PGA. The affinities of heavy metal ions for γ-PGA followed the order: $Cu^{2+} > Zn^{2+} > Ni^{2+} > Cd^{2+}$. Copper (II) adsorption capacity of γ-PGA strongly depended on its molecular weight as high molecular weight γ-PGA is more effective for metal removal than low molecular weight γ-PGA. Isotherm models showed that the Redlich-Peterson best described the metal adsorption capacity of γ-PGA. It was also found that a multisite adsorption mechanism occurred via the complexation of metal ions with the free α-carboxyl and possibly the amide functional groups in γ-PGA.

Overall, it can be concluded that high molecular weight γ-PGA exhibits high metal binding ability and can be a potential adsorbent for removing heavy metal ions such as Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+} and Ag^+ from water and wastewater. Furthermore, since this biopolymer is non-toxic and biodegradable, it can offer green solution to water and waste water treatment plants as well as in various industries utilizing heavy metals. The surrounding environment will also

benefit from the eradication of potential toxicity of heavy metals as well as recovery of precious metals.

9.0 FUTURE WORK

Even though a number of researches had been previously carried out in this field, the results obtained in this study bring up opportunities for future research.

This investigation showed that even though all the bacterial strains used in this study are thermotolerant, they are not truly thermophilic because their growth was not sustained and γ-PGA was not produced at 50°C. It would therefore, be interesting to investigate the growth and γ-PGA production of these strains at a temperature between 37^{o}C and 50^{o}C with time for a more cost effective γ-PGA production.

It was found that pH and aeration control are important for growth and γ-PGA production and that optimum pH depends on the bacterial strain. The scale up of γ-PGA production in a high aerated and pH controlled fermenter would be important for efficient production of γ-PGA.

Two *Bacillus* strains, *B. licheniformis* 1525 and NCTC 6816 even though they produced γ-PGA in all investigated media (GS, C and E), the yields of γ-PGA were relatively low compared to the other strains investigated in this study. It could be that these strains generally have low metabolic synthesizing capability since other strains under the same condition had better yields and may require specific nutritional needs for optimal γ-PGA production. Therefore, it would be worth investigating the effects of growth parameters and culture conditions on the production of γ-PGA by *B. licheniformis* 1525 and NCTC 6816 in order to confirm whether they are capable of producing higher yields of γ-PGA.

It was observed that the presence of either low (0.5%) NaCl concentration or citric acid + low (0.5%) NaCl concentration in the culture medium strongly influenced the molecular weight of γ-PGA suggesting that the presence of either low (0.5%) NaCl concentration or both citric acid and low NaCl concentration decreased the activity of degradation enzymes. The influence of medium composition on the activity of degradation enzymes in γ-PGA production by *B. licheniformis* ATCC 9945a and *B. subtilis* (natto) ATCC 15245 would thus

need to be investigated in order to confirm the role of citric acid + low concentration of NaCl in the inactivation of these enzymes and/or elongation of γ-PGA.

It was also observed that even though the amount of metal ion adsorbed increased with increasing pH values before metal ions started to precipitate, the adsorption capacity of γ-PGA in a non-pH controlled system was higher than in a pH controlled system in our study which might be due to the presence of interfering Na^+ from NaOH on the mechanism of adsorption. Therefore, further investigation needs to be carried out in order to confirm this possibility and perhaps consider the use of alternative acid neutralisers.

As γ-PGA is capable of adsorbing heavy metals, the desorption of adsorbed heavy metals from used up γ-PGA is also possible (Inbaraj *et al*., 2009). According to their report, a maximum mercury (II) desorption of 82.5% was attained when distilled water adjusted to pH 1 with HNO_3 was used as desorbing agent while the highest mercury (II) recovery of 98.8% was achieved with distilled water adjusted to pH 2 by HCl. It would be interesting to investigate the desorption of all metal ions investigated in this study from spent γ-PGA and re-usability of the recovered γ-PGA which would reduce treatment cost efficiently and make possible the recovery of precious metals such as silver.

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11.0 APPENDICES

11.1: Two-way ANOVA result showing the effect of temperature (37^{o}C) with time on growth of *B. licheniformis 1525*

Table Analyzed	*Bacillus licheniformis* 1525 37^{o}C
Two-way ANOVA	Ordinary

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 hour					
GS medium vs. Medium C	0.0	-0.1058 to 0.1058	No	ns	> 0.9999
GS medium vs. Medium E	-0.02000	-0.1258 to 0.08576	No	ns	> 0.9999
Medium C vs. Medium E	-0.02000	-0.1258 to 0.08576	No	ns	> 0.9999
24 hours					
GS medium vs. Medium C	0.03500	-0.07076 to 0.1408	No	ns	> 0.9999
GS medium vs. Medium E	0.3050	0.1992 to 0.4108	Yes	****	< 0.0001
Medium C vs. Medium E	0.2700	0.1642 to 0.3758	Yes	****	< 0.0001
48 hours					
GS medium vs. Medium C	0.07000	-0.03576 to 0.1758	No	ns	0.6487
GS medium vs. Medium E	0.03000	-0.07576 to 0.1358	No	ns	> 0.9999
Medium C vs. Medium E	-0.04000	-0.1458 to 0.06576	No	ns	> 0.9999
72 hours					
GS medium vs. Medium C	0.06000	-0.04576 to 0.1658	No	ns	> 0.9999
GS medium vs. Medium E	-0.2450	-0.3508 to -0.1392	Yes	****	< 0.0001
Medium C vs. Medium E	-0.3050	-0.4108 to -0.1992	Yes	****	< 0.0001
96 hours					
GS medium vs. Medium C	-0.01000	-0.1158 to 0.09576	No	ns	> 0.9999
GS medium vs. Medium E	-0.3100	-0.4158 to -0.2042	Yes	****	< 0.0001
Medium C vs. Medium E	-0.3000	-0.4058 to -0.1942	Yes	****	< 0.0001

11.2: Two-way ANOVA result showing the effect of temperature (50°C) with time on growth of *B. licheniformis 1525*

Table Analyzed	*Bacillus licheniformis* 1525 50°C
Two-way ANOVA	Ordinary

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 hour					
GS medium vs. Medium C	-0.01000	-0.1682 to 0.1482	No	ns	> 0.9999
GS medium vs. Medium E	0.0	-0.1582 to 0.1582	No	ns	> 0.9999
Medium C vs. Medium E	0.01000	-0.1482 to 0.1682	No	ns	> 0.9999
24 hours					
GS medium vs. Medium C	-0.01000	-0.1682 to 0.1482	No	ns	> 0.9999
GS medium vs. Medium E	0.0	-0.1582 to 0.1582	No	ns	> 0.9999
Medium C vs. Medium E	0.01000	-0.1482 to 0.1682	No	ns	> 0.9999
48 hours					
GS medium vs. Medium C	-0.04500	-0.2032 to 0.1132	No	ns	> 0.9999
GS medium vs. Medium E	-0.08500	-0.2432 to 0.07316	No	ns	> 0.9999
Medium C vs. Medium E	-0.04000	-0.1982 to 0.1182	No	ns	> 0.9999
72 hours					
GS medium vs. Medium C	-0.1150	-0.2732 to 0.04316	No	ns	0.4112
GS medium vs. Medium E	-0.3550	-0.5132 to -0.1968	Yes	****	< 0.0001
Medium C vs. Medium E	-0.2400	-0.3982 to -0.08184	Yes	***	0.0006
96 hours					
GS medium vs. Medium C	-0.1600	-0.3182 to -0.001840	Yes	*	0.0454
GS medium vs. Medium E	-0.4200	-0.5782 to -0.2618	Yes	****	< 0.0001
Medium C vs. Medium E	-0.2600	-0.4182 to -0.1018	Yes	***	0.0002

11.3: Two-way ANOVA result showing the effect of different temperatures (37 and 50°C) on the growth of B. *licheniformis 1525* in different media.

Table Analyzed	*Bacillus licheniformis* 1525
Two-way ANOVA	Ordinary

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
GS medium (37°C - 50°C)					
0 hour	0.0	-0.08123 to 0.08123	No	ns	> 0.9999
24 hours	0.4150	0.3338 to 0.4962	Yes	****	< 0.0001
48 hours	1.405	1.324 to 1.486	Yes	****	< 0.0001
72 hours	1.875	1.794 to 1.956	Yes	****	< 0.0001
96 hours	1.660	1.579 to 1.741	Yes	****	< 0.0001
Medium C (37°C - 50°C)					
0 hour	-0.01000	-0.1754 to 0.1554	No	ns	> 0.9999
24 hours	0.3700	0.2046 to 0.5354	Yes	****	< 0.0001
48 hours	1.290	1.125 to 1.455	Yes	****	< 0.0001
72 hours	1.700	1.535 to 1.865	Yes	****	< 0.0001
96 hours	1.510	1.345 to 1.675	Yes	****	< 0.0001
Medium E (37°C - 50°C)					
0 hour	0.02000	-0.07628 to 0.1163	No	ns	> 0.9999
24 hours	0.1100	0.01372 to 0.2063	Yes	*	0.0200
48 hours	1.290	1.194 to 1.386	Yes	****	< 0.0001
72 hours	1.765	1.669 to 1.861	Yes	****	< 0.0001
96 hours	1.550	1.454 to 1.646	Yes	****	< 0.0001

11.4: Two-way ANOVA result showing the effect of temperature (37°C) with time on growth of *B. licheniformis* NCTC 6816

Table Analyzed	*Bacillus licheniformis* NCTC 6816 37°C
Two-way ANOVA	Ordinary

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 hour					
GS medium vs. Medium C	0.01000	-0.1213 to 0.1413	No	ns	> 0.9999
GS medium vs. Medium E	0.005000	-0.1263 to 0.1363	No	ns	> 0.9999
Medium C vs. Medium E	-0.005000	-0.1363 to 0.1263	No	ns	> 0.9999
24 hours					
GS medium vs. Medium C	-0.02000	-0.1513 to 0.1113	No	ns	> 0.9999
GS medium vs. Medium E	0.2250	0.09365 to 0.3563	Yes	****	< 0.0001
Medium C vs. Medium E	0.2450	0.1137 to 0.3763	Yes	****	< 0.0001
48 hours					
GS medium vs. Medium C	-0.01500	-0.1463 to 0.1163	No	ns	> 0.9999
GS medium vs. Medium E	-0.07000	-0.2013 to 0.06135	No	ns	> 0.9999
Medium C vs. Medium E	-0.05500	-0.1863 to 0.07635	No	ns	> 0.9999
72 hours					
GS medium vs. Medium C	0.009999	-0.1213 to 0.1413	No	ns	> 0.9999
GS medium vs. Medium E	-0.2500	-0.3813 to -0.1187	Yes	****	< 0.0001
Medium C vs. Medium E	-0.2600	-0.3913 to -0.1287	Yes	****	< 0.0001
96 hours					
GS medium vs. Medium C	-0.02500	-0.1563 to 0.1063	No	ns	> 0.9999
GS medium vs. Medium E	-0.3450	-0.4763 to -0.2137	Yes	****	< 0.0001
Medium C vs. Medium E	-0.3200	-0.4513 to -0.1887	Yes	****	< 0.0001

11.5: Two-way ANOVA result showing the effect of temperature (50°C) with time on growth of *B. licheniformis* NCTC 6816

Table Analyzed	*Bacillus licheniformis* NCTC 6816 50°C
Two-way ANOVA	Ordinary

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 hour					
GS medium vs. Medium C	0.0	-0.1645 to 0.1645	No	ns	> 0.9999
GS medium vs. Medium E	0.01000	-0.1545 to 0.1745	No	ns	> 0.9999
Medium C vs. Medium E	0.01000	-0.1545 to 0.1745	No	ns	> 0.9999
24 hours					
GS medium vs. Medium C	0.03000	-0.1345 to 0.1945	No	ns	> 0.9999
GS medium vs. Medium E	-0.08000	-0.2445 to 0.08446	No	ns	> 0.9999
Medium C vs. Medium E	-0.1100	-0.2745 to 0.05446	No	ns	0.6186
48 hours					
GS medium vs. Medium C	0.07000	-0.09446 to 0.2345	No	ns	> 0.9999
GS medium vs. Medium E	-0.05000	-0.2145 to 0.1145	No	ns	> 0.9999
Medium C vs. Medium E	-0.1200	-0.2845 to 0.04446	No	ns	0.4038
72 hours					
GS medium vs. Medium C	-0.04000	-0.2045 to 0.1245	No	ns	> 0.9999
GS medium vs. Medium E	-0.08500	-0.2495 to 0.07946	No	ns	> 0.9999
Medium C vs. Medium E	-0.04500	-0.2095 to 0.1195	No	ns	> 0.9999
96 hours					
GS medium vs. Medium C	0.03000	-0.1345 to 0.1945	No	ns	> 0.9999
GS medium vs. Medium E	0.08500	-0.07946 to 0.2495	No	ns	> 0.9999
Medium C vs. Medium E	0.05500	-0.1095 to 0.2195	No	ns	> 0.9999

11.6: Two-way ANOVA result showing the effect of different temperatures (37 and 50°C) on the growth of *B. licheniformis* NCTC 6816 in different media.

Table Analyzed	*Bacillus licheniformis* NCTC 6816
Two-way ANOVA	Ordinary

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
GS medium (37 °C - 50 °C)					
0 hour	-0.01000	-0.1304 to 0.1104	No	ns	> 0.9999
24 hours	0.4200	0.2996 to 0.5404	Yes	****	< 0.0001
48 hours	0.9850	0.8646 to 1.105	Yes	****	< 0.0001
72 hours	1.380	1.260 to 1.500	Yes	****	< 0.0001
96 hours	1.435	1.315 to 1.555	Yes	****	< 0.0001
Medium C (37 °C - 50 °C)					
0 hour	-0.02000	-0.1667 to 0.1267	No	ns	> 0.9999
24 hours	0.4700	0.3233 to 0.6167	Yes	****	< 0.0001
48 hours	1.070	0.9233 to 1.217	Yes	****	< 0.0001
72 hours	1.330	1.183 to 1.477	Yes	****	< 0.0001
96 hours	1.490	1.343 to 1.637	Yes	****	< 0.0001
Medium E (37 °C - 50 °C)					
0 hour	-0.005000	-0.1349 to 0.1249	No	ns	> 0.9999
24 hours	0.1150	-0.01492 to 0.2449	No	ns	0.1020
48 hours	1.005	0.8751 to 1.135	Yes	****	< 0.0001
72 hours	1.545	1.415 to 1.675	Yes	****	< 0.0001
96 hours	1.865	1.735 to 1.995	Yes	****	< 0.0001

11.7: Two-way ANOVA result showing the effect of temperature (37°C) with time on growth of *B. licheniformis* ATCC 9945a

Table Analyzed	*Bacillus licheniformis* ATCC 9945a 37°C
Two-way ANOVA	Ordinary

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 hour					
GS medium vs. Medium C	0.0	-0.1062 to 0.1062	No	ns	> 0.9999
GS medium vs. Medium E	0.0	-0.1062 to 0.1062	No	ns	> 0.9999
Medium C vs. Medium E	0.0	-0.1062 to 0.1062	No	ns	> 0.9999
24 hours					
GS medium vs. Medium C	0.02400	-0.08215 to 0.1302	No	ns	> 0.9999
GS medium vs. Medium E	0.06000	-0.04615 to 0.1662	No	ns	> 0.9999
Medium C vs. Medium E	0.03600	-0.07015 to 0.1422	No	ns	> 0.9999
48 hours					
GS medium vs. Medium C	0.02300	-0.08315 to 0.1292	No	ns	> 0.9999
GS medium vs. Medium E	-0.01700	-0.1232 to 0.08915	No	ns	> 0.9999
Medium C vs. Medium E	-0.04000	-0.1462 to 0.06615	No	ns	> 0.9999
72 hours					
GS medium vs. Medium C	0.02300	-0.08315 to 0.1292	No	ns	> 0.9999
GS medium vs. Medium E	0.1430	0.03685 to 0.2492	Yes	**	0.0025
Medium C vs. Medium E	0.1200	0.01385 to 0.2262	Yes	*	0.0168
96 hours					
GS medium vs. Medium C	0.09700	-0.009151 to 0.2032	No	ns	0.1003
GS medium vs. Medium E	0.1700	0.06385 to 0.2762	Yes	***	0.0003
Medium C vs. Medium E	0.07300	-0.03315 to 0.1792	No	ns	0.5430

11.8: Two-way ANOVA result showing the effect of temperature (50°C) with time on growth of *B. licheniformis* ATCC 9945a

Table Analyzed	*Bacillus licheniformis* ATCC 9945a 50°C
Two-way ANOVA	Ordinary

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 hour					
GS medium vs. Medium C	-0.04000	-0.2061 to 0.1261	No	ns	> 0.9999
GS medium vs. Medium E	0.0	-0.1661 to 0.1661	No	ns	> 0.9999
Medium C vs. Medium E	0.04000	-0.1261 to 0.2061	No	ns	> 0.9999
24 hours					
GS medium vs. Medium C	-0.005000	-0.1711 to 0.1611	No	ns	> 0.9999
GS medium vs. Medium E	0.01500	-0.1511 to 0.1811	No	ns	> 0.9999
Medium C vs. Medium E	0.02000	-0.1461 to 0.1861	No	ns	> 0.9999
48 hours					
GS medium vs. Medium C	0.06000	-0.1061 to 0.2261	No	ns	> 0.9999
GS medium vs. Medium E	0.1000	-0.06610 to 0.2661	No	ns	0.9666
Medium C vs. Medium E	0.04000	-0.1261 to 0.2061	No	ns	> 0.9999
72 hours					
GS medium vs. Medium C	0.04000	-0.1261 to 0.2061	No	ns	> 0.9999
GS medium vs. Medium E	0.1300	-0.03610 to 0.2961	No	ns	0.2745
Medium C vs. Medium E	0.09000	-0.07610 to 0.2561	No	ns	> 0.9999
96 hours					
GS medium vs. Medium C	0.06000	-0.1061 to 0.2261	No	ns	> 0.9999
GS medium vs. Medium E	0.1500	-0.01610 to 0.3161	No	ns	0.1092
Medium C vs. Medium E	0.09000	-0.07610 to 0.2561	No	ns	> 0.9999

11.9: Two-way ANOVA result showing the effect of different temperatures (37 and 50^{o}C) on the growth of *B. licheniformis* ATCC 9945a in different media.

Table Analyzed	*Bacillus licheniformis* ATCC 9945a
Two-way ANOVA	Ordinary

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
GS medium (37 ^{o}C - 50 ^{o}C)					
0 hour	0.007000	-0.1139 to 0.1279	No	ns	> 0.9999
24 hours	1.117	0.9961 to 1.238	Yes	****	< 0.0001
48 hours	1.453	1.332 to 1.574	Yes	****	< 0.0001
72 hours	1.683	1.562 to 1.804	Yes	****	< 0.0001
96 hours	1.837	1.716 to 1.958	Yes	****	< 0.0001
Medium C (37 ^{o}C - 50 ^{o}C)					
0 hour	-0.03300	-0.1216 to 0.05559	No	ns	> 0.9999
24 hours	1.088	0.9994 to 1.177	Yes	****	< 0.0001
48 hours	1.490	1.401 to 1.579	Yes	****	< 0.0001
72 hours	1.700	1.611 to 1.789	Yes	****	< 0.0001
96 hours	1.800	1.711 to 1.889	Yes	****	< 0.0001
Medium E (37 ^{o}C - 50 ^{o}C)					
0 hour	0.007000	-0.1478 to 0.1618	No	ns	> 0.9999
24 hours	1.072	0.9172 to 1.227	Yes	****	< 0.0001
48 hours	1.570	1.415 to 1.725	Yes	****	< 0.0001
72 hours	1.670	1.515 to 1.825	Yes	****	< 0.0001
96 hours	1.817	1.662 to 1.972	Yes	****	< 0.0001

11.10: Two-way ANOVA result showing the effect of temperature (37°C) with time on growth of *B. licheniformis* ATCC 9945

Table Analyzed	*B. licheniformis* ATCC 9945 37°C				
Two-way ANOVA	Ordinary				
Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 hour					
GS medium vs. Medium C	0.02000	-0.06189 to 0.1019	No	ns	> 0.9999
GS medium vs. Medium E	0.01300	-0.06889 to 0.09489	No	ns	> 0.9999
Medium C vs. Medium E	-0.007000	-0.08889 to 0.07489	No	ns	> 0.9999
24 hours					
GS medium vs. Medium C	0.006000	-0.07589 to 0.08789	No	ns	> 0.9999
GS medium vs. Medium E	-0.04700	-0.1289 to 0.03489	No	ns	0.4679
Medium C vs. Medium E	-0.05300	-0.1349 to 0.02889	No	ns	0.3337
48 hours					
GS medium vs. Medium C	0.07300	-0.008892 to 0.1549	No	ns	0.0936
GS medium vs. Medium E	-0.02000	-0.1019 to 0.06189	No	ns	> 0.9999
Medium C vs. Medium E	-0.09300	-0.1749 to -0.01111	Yes	*	0.0218
72 hours					
GS medium vs. Medium C	0.1070	0.02511 to 0.1889	Yes	**	0.0072
GS medium vs. Medium E	-0.5330	-0.6149 to -0.4511	Yes	****	< 0.0001
Medium C vs. Medium E	-0.6400	-0.7219 to -0.5581	Yes	****	< 0.0001
96 hours					
GS medium vs. Medium C	0.09400	0.01211 to 0.1759	Yes	*	0.0202
GS medium vs. Medium E	-0.4860	-0.5679 to -0.4041	Yes	****	< 0.0001
Medium C vs. Medium E	-0.5800	-0.6619 to -0.4981	Yes	****	< 0.0001

11.11: Two-way ANOVA result showing the effect of temperature (50°C) with time on growth of *B. licheniformis* ATCC 9945

Table Analyzed	*B. licheniformis* ATCC 9945 50°C
Two-way ANOVA	Ordinary

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 hour					
GS medium vs. Medium C	0.02000	-0.05052 to 0.09052	No	ns	> 0.9999
GS medium vs. Medium E	-0.02000	-0.09052 to 0.05052	No	ns	> 0.9999
Medium C vs. Medium E	-0.04000	-0.1105 to 0.03052	No	ns	0.4820
24 hours					
GS medium vs. Medium C	0.01500	-0.05552 to 0.08552	No	ns	> 0.9999
GS medium vs. Medium E	-0.005000	-0.07552 to 0.06552	No	ns	> 0.9999
Medium C vs. Medium E	-0.02000	-0.09052 to 0.05052	No	ns	> 0.9999
48 hours					
GS medium vs. Medium C	0.08000	0.009484 to 0.1505	Yes	*	0.0220
GS medium vs. Medium E	-0.02000	-0.09052 to 0.05052	No	ns	> 0.9999
Medium C vs. Medium E	-0.1000	-0.1705 to -0.02948	Yes	**	0.0034
72 hours					
GS medium vs. Medium C	0.02500	-0.04552 to 0.09552	No	ns	> 0.9999
GS medium vs. Medium E	-0.08500	-0.1555 to -0.01448	Yes	*	0.0140
Medium C vs. Medium E	-0.1100	-0.1805 to -0.03948	Yes	**	0.0013
96 hours					
GS medium vs. Medium C	0.03000	-0.04052 to 0.1005	No	ns	0.8678
GS medium vs. Medium E	0.03500	-0.03552 to 0.1055	No	ns	0.6536
Medium C vs. Medium E	0.005000	-0.06552 to 0.07552	No	ns	> 0.9999

11.12: Two-way ANOVA result showing the effect of different temperatures (37 and 50^{o}C) on the growth of *B. licheniformis* ATCC 9945 in different media.

Table Analyzed	*Bacillus licheniformis* ATCC 9945
Two-way ANOVA	Ordinary

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
GS medium (37 ^{o}C - 50 ^{o}C)					
0 hour	0.3330	0.2409 to 0.4251	Yes	****	< 0.0001
24 hours	0.9230	0.8309 to 1.015	Yes	****	< 0.0001
48 hours	1.373	1.281 to 1.465	Yes	****	< 0.0001
72 hours	1.135	1.043 to 1.227	Yes	****	< 0.0001
96 hours	1.357	1.265 to 1.449	Yes	****	< 0.0001
Medium C (37 ^{o}C - 50 ^{o}C)					
0 hour	0.3330	0.2398 to 0.4262	Yes	****	< 0.0001
24 hours	0.9320	0.8388 to 1.025	Yes	****	< 0.0001
48 hours	1.380	1.287 to 1.473	Yes	****	< 0.0001
72 hours	1.053	0.9598 to 1.146	Yes	****	< 0.0001
96 hours	1.293	1.200 to 1.386	Yes	****	< 0.0001
Medium E (37 ^{o}C - 50 ^{o}C)					
0 hour	0.3000	0.2301 to 0.3699	Yes	****	< 0.0001
24 hours	0.9650	0.8951 to 1.035	Yes	****	< 0.0001
48 hours	1.373	1.303 to 1.443	Yes	****	< 0.0001
72 hours	1.583	1.513 to 1.653	Yes	****	< 0.0001
96 hours	1.878	1.808 to 1.948	Yes	****	< 0.0001

11.13: Two-way ANOVA result showing the effect of temperature (37°C) with time on growth of *B. subtilis* (natto) ATCC 15245

Table Analyzed	*B. subtilis* (natto) ATCC 15245 37°C
Two-way ANOVA	Ordinary

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 hour					
GS medium vs. Medium C	-0.01700	-0.2191 to 0.1851	No	ns	> 0.9999
GS medium vs. Medium E	-0.01700	-0.2191 to 0.1851	No	ns	> 0.9999
Medium C vs. Medium E	0.0	-0.2021 to 0.2021	No	ns	> 0.9999
24 hours					
GS medium vs. Medium C	0.09600	-0.1061 to 0.2981	No	ns	0.7134
GS medium vs. Medium E	0.1230	-0.07910 to 0.3251	No	ns	0.3998
Medium C vs. Medium E	0.02700	-0.1751 to 0.2291	No	ns	> 0.9999
48 hours					
GS medium vs. Medium C	0.01000	-0.1921 to 0.2121	No	ns	> 0.9999
GS medium vs. Medium E	-0.6170	-0.8191 to -0.4149	Yes	****	< 0.0001
Medium C vs. Medium E	-0.6270	-0.8291 to -0.4249	Yes	****	< 0.0001
72 hours					
GS medium vs. Medium C	0.03000	-0.1721 to 0.2321	No	ns	> 0.9999
GS medium vs. Medium E	-0.2100	-0.4121 to -0.007899	Yes	*	0.0396
Medium C vs. Medium E	-0.2400	-0.4421 to -0.03790	Yes	*	0.0157
96 hours					
GS medium vs. Medium C	-0.08000	-0.2821 to 0.1221	No	ns	0.9706
GS medium vs. Medium E	0.1430	-0.05910 to 0.3451	No	ns	0.2486
Medium C vs. Medium E	0.2230	0.02090 to 0.4251	Yes	*	0.0267

11.14: Two-way ANOVA result showing the effect of temperature (50°C) with time on growth of *B. subtilis* (natto) ATCC 15245

Table Analyzed	*B. subtilis* (natto) ATCC 15245 50°C
Two-way ANOVA	Ordinary

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
0 hour					
GS medium vs. Medium C	0.0	-0.1127 to 0.1127	No	ns	> 0.9999
GS medium vs. Medium E	0.01600	-0.09672 to 0.1287	No	ns	> 0.9999
Medium C vs. Medium E	0.01600	-0.09672 to 0.1287	No	ns	> 0.9999
24 hours					
GS medium vs. Medium C	-0.02000	-0.1327 to 0.09272	No	ns	> 0.9999
GS medium vs. Medium E	0.06000	-0.05272 to 0.1727	No	ns	0.5616
Medium C vs. Medium E	0.08000	-0.03272 to 0.1927	No	ns	0.2460
48 hours					
GS medium vs. Medium C	-0.03700	-0.1497 to 0.07572	No	ns	> 0.9999
GS medium vs. Medium E	0.3100	0.1973 to 0.4227	Yes	****	< 0.0001
Medium C vs. Medium E	0.3470	0.2343 to 0.4597	Yes	****	< 0.0001
72 hours					
GS medium vs. Medium C	0.1070	-0.005725 to 0.2197	No	ns	0.0673
GS medium vs. Medium E	0.1870	0.07428 to 0.2997	Yes	***	0.0006
Medium C vs. Medium E	0.08000	-0.03272 to 0.1927	No	ns	0.2460
96 hours					
GS medium vs. Medium C	0.0	-0.1127 to 0.1127	No	ns	> 0.9999
GS medium vs. Medium E	0.1400	0.02728 to 0.2527	Yes	*	0.0111
Medium C vs. Medium E	0.1400	0.02728 to 0.2527	Yes	*	0.0111

11.15: Two-way ANOVA result showing the effect of different temperatures (37 and 50^{o}C) on the growth of *B. subtilis* (natto) ATCC 15245 in different media.

Table Analyzed	*B. subtilis* (natto) ATCC 15245
Two-way ANOVA	Ordinary

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
GS medium (37 ^{o}C - 50 ^{o}C)					
0 hour	-0.5300	-0.6955 to -0.3645	Yes	****	< 0.0001
24 hours	1.166	1.001 to 1.331	Yes	****	< 0.0001
48 hours	0.9330	0.7675 to 1.098	Yes	****	< 0.0001
72 hours	1.200	1.035 to 1.365	Yes	****	< 0.0001
96 hours	1.650	1.485 to 1.815	Yes	****	< 0.0001
Medium C (37 ^{o}C - 50 ^{o}C)					
0 hour	-0.5130	-0.7578 to -0.2682	Yes	****	< 0.0001
24 hours	1.050	0.8052 to 1.295	Yes	****	< 0.0001
48 hours	0.8860	0.6412 to 1.131	Yes	****	< 0.0001
72 hours	1.277	1.032 to 1.522	Yes	****	< 0.0001
96 hours	1.730	1.485 to 1.975	Yes	****	< 0.0001
Medium E (37 ^{o}C - 50 ^{o}C)					
0 hour	-0.4970	-0.6146 to -0.3794	Yes	****	< 0.0001
24 hours	1.103	0.9854 to 1.221	Yes	****	< 0.0001
48 hours	1.860	1.742 to 1.978	Yes	****	< 0.0001
72 hours	1.597	1.479 to 1.715	Yes	****	< 0.0001
96 hours	1.647	1.529 to 1.765	Yes	****	< 0.0001

11.16a: Two-way ANOVA result showing the effect of culture media on γ-PGA production

Table Analyzed	Culture media
Two-way ANOVA	Ordinary

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
Bacillus licheniformis 1525					
GS medium vs. Medium C	-0.04000	-1.996 to 1.916	No	ns	> 0.9999
GS medium vs. Medium E	0.2000	-1.756 to 2.156	No	ns	> 0.9999
Medium C vs. Medium E	0.2400	-1.716 to 2.196	No	ns	> 0.9999
Bacillus licheniformis NCTC 6816					
GS medium vs. Medium C	-0.1870	-2.143 to 1.769	No	ns	> 0.9999
GS medium vs. Medium E	0.2670	-1.689 to 2.223	No	ns	> 0.9999
Medium C vs. Medium E	0.4540	-1.502 to 2.410	No	ns	> 0.9999
Bacillus licheniformis 9945a					
GS medium vs. Medium C	-0.4200	-2.376 to 1.536	No	ns	> 0.9999
GS medium vs. Medium E	0.6800	-1.276 to 2.636	No	ns	> 0.9999
Medium C vs. Medium E	1.100	-0.8559 to 3.056	No	ns	> 0.9999
Bacillus licheniformis 9945					
GS medium vs. Medium C	0.0	-1.956 to 1.956	No	ns	> 0.9999
GS medium vs. Medium E	-8.733	-10.69 to -6.777	Yes	****	< 0.0001
Medium C vs. Medium E	-8.733	-10.69 to -6.777	Yes	****	< 0.0001
Bacillus subtilis (natto) 15245					
GS medium vs. Medium C	0.1000	-1.856 to 2.056	No	ns	> 0.9999
GS medium vs. Medium E	5.650	3.694 to 7.606	Yes	****	< 0.0001
Medium C vs. Medium E	5.550	3.594 to 7.506	Yes	****	< 0.0001

11.16b: Two-way ANOVA result showing the effect of culture media on γ-PGA production

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
GS medium					
Bacillus licheniformis 1525 vs. *Bacillus licheniformis* NCTC 6816	0.7070	-1.412 to 2.826	No	ns	> 0.9999
Bacillus licheniformis 1525 vs. *Bacillus licheniformis* 9945a	-6.123	-8.242 to -4.004	Yes	****	< 0.0001
Bacillus licheniformis 1525 vs. *Bacillus licheniformis* 9945	4.827	2.708 to 6.946	Yes	****	< 0.0001
Bacillus licheniformis 1525 vs. *Bacillus subtilis* (natto) 15245	-6.863	-8.982 to -4.744	Yes	****	< 0.0001
Bacillus licheniformis NCTC 6816 vs. *Bacillus licheniformis* 9945a	-6.830	-8.949 to -4.711	Yes	****	< 0.0001
Bacillus licheniformis NCTC 6816 vs. *Bacillus licheniformis* 9945	4.120	2.001 to 6.239	Yes	****	< 0.0001
Bacillus licheniformis NCTC 6816 vs. *Bacillus subtilis* (natto) 15245	-7.570	-9.689 to -5.451	Yes	****	< 0.0001
Bacillus licheniformis 9945a vs. *Bacillus licheniformis* 9945	10.95	8.831 to 13.07	Yes	****	< 0.0001
Bacillus licheniformis 9945a vs. *Bacillus subtilis* (natto) 15245	-0.7400	-2.859 to 1.379	No	ns	> 0.9999
Bacillus licheniformis 9945 vs. *Bacillus subtilis* (natto) 15245	-11.69	-13.81 to -9.571	Yes	****	< 0.0001
Medium C					
Bacillus licheniformis 1525 vs. *Bacillus licheniformis* NCTC 6816	0.5600	-1.559 to 2.679	No	ns	> 0.9999
Bacillus licheniformis 1525 vs. *Bacillus licheniformis* 9945a	-6.503	-8.622 to -4.384	Yes	****	< 0.0001
Bacillus licheniformis 1525 vs. *Bacillus licheniformis* 9945	4.867	2.748 to 6.986	Yes	****	< 0.0001
Bacillus licheniformis 1525 vs. *Bacillus subtilis* (natto) 15245	-6.723	-8.842 to -4.604	Yes	****	< 0.0001
Bacillus licheniformis NCTC 6816 vs. *Bacillus licheniformis* 9945a	-7.063	-9.182 to -4.944	Yes	****	< 0.0001
Bacillus licheniformis NCTC 6816 vs. *Bacillus licheniformis* 9945	4.307	2.188 to 6.426	Yes	****	< 0.0001
Bacillus licheniformis NCTC 6816 vs. *Bacillus subtilis* (natto) 15245	-7.283	-9.402 to -5.164	Yes	****	< 0.0001
Bacillus licheniformis 9945a vs. *Bacillus licheniformis* 9945	11.37	9.251 to 13.49	Yes	****	< 0.0001
Bacillus licheniformis 9945a vs. *Bacillus subtilis* (natto) 15245	-0.2200	-2.339 to 1.899	No	ns	> 0.9999
Bacillus licheniformis 9945 vs. *Bacillus subtilis* (natto) 15245	-11.59	-13.71 to -9.471	Yes	****	< 0.0001

Medium E					
Bacillus licheniformis 1525 vs. *Bacillus licheniformis* NCTC 6816	0.7740	-1.345 to 2.893	No	ns	> 0.9999
Bacillus licheniformis 1525 vs. *Bacillus licheniformis* 9945a	-5.643	-7.762 to -3.524	Yes	****	< 0.0001
Bacillus licheniformis 1525 vs. *Bacillus licheniformis* 9945	-4.106	-6.225 to -1.987	Yes	****	< 0.0001
Bacillus licheniformis 1525 vs. *Bacillus subtilis* (natto) 15245	-1.413	-3.532 to 0.7058	No	ns	0.8505
Bacillus licheniformis NCTC 6816 vs. *Bacillus licheniformis* 9945a	-6.417	-8.536 to -4.298	Yes	****	< 0.0001
Bacillus licheniformis NCTC 6816 vs. *Bacillus licheniformis* 9945	-4.880	-6.999 to -2.761	Yes	****	< 0.0001
Bacillus licheniformis NCTC 6816 vs. *Bacillus subtilis* (natto) 15245	-2.187	-4.306 to -0.06818	Yes	*	0.0372
Bacillus licheniformis 9945a vs. *Bacillus licheniformis* 9945	1.537	-0.5818 to 3.656	No	ns	0.5362
Bacillus licheniformis 9945a vs. *Bacillus subtilis* (natto) 15245	4.230	2.111 to 6.349	Yes	****	< 0.0001
Bacillus licheniformis 9945 vs. *Bacillus subtilis* (natto) 15245	2.693	0.5742 to 4.812	Yes	**	0.0039

LIST OF PUBLICATIONS AND CONFERENCE PAPERS

Publication

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Poly-γ-glutamic acid: production, properties and applications

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Abstract

Poly-γ-glutamic acid (γ-PGA) is a naturally occurring biopolymer made up of repeating units of L-glutamic acid, D-glutamic acid or both. γ-PGA can exhibit different properties (conformational states, enantiomeric properties and molecular mass). Owing to its biodegradable, non-toxic and non-immunogenic properties, it has been used successfully in the food, medical and wastewater industries. Amongst other novel applications, it has the potential to be used for protein crystallization, as a soft tissue adhesive and a non-viral vector for safe gene delivery. This review focuses on the production, properties and applications of γ-PGA. Each application of γ-PGA utilizes specific properties attributed to various forms of γ-PGA. As a result of its growing applications, more strains of bacteria need to be investigated for γ-PGA production to obtain high yields of γ-PGA with different properties. Many medical applications (especially drug delivery) have exploited α-PGA. As γ-PGA is essentially different from α-PGA (i.e. it does not involve a chemical modification step and is not susceptible to proteases), it could be better utilized for such medical applications. Optimization of γ-PGA with respect to cost of production, molecular mass and conformational/enantiomeric properties is a major step in making its application practical. Analyses of γ-PGA production and knowledge of the enzymes and genes involved in γ-PGA production will not only help increase productivity whilst reducing the cost of production, but also help to understand the mechanism by which γ-PGA is effective in numerous applications.

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Abbreviations:
GGT
γ-glutamyl-transpeptidase
GPC
gel permeation chromatography
NK
natural killer
NP
nanoparticle
PEC
polyelectrolyte complex
PGA
polyglutamic acid